# CHARACTERISATION OF EPIDERMAL GROWTH FACTOR RECEPTOR (EGF-R) IN THE HUMAN PROSTATE

By

Samuel Quarcoo Maddy, FIMLS, MSc

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# CONTENTS

# UNIVERSITY OF EDINBURGH

## ABSTRACT OF THESIS (Regulation 7.9)

*Name of Candidate* Samuel Quarcoo Maddy

*Address* 3FR 16 Bristo Place, Edinburgh EH1 1EZ

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*Title of Thesis* Characterisation of Epidermal Growth Factor Receptors in the Human Prostate

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Epidermal growth factor (EGF) receptors have been localised and characterised in the human benign prostatic hyperplasia (BPH). The receptors were expressed in more than 85% of BPH tissues examined.

Binding studies showed that optimal uptake of $^{125}$I-EGF was achieved within 60-90 minutes at 37°C. Other binding characteristics demonstrated the saturability of the receptor sites and also revealed two classes of binding sites of higher (mean dissociation constant Kd ± SD = 0.8 ± 0.2 nmol/l) and lower (Kd ± SD = 7.6 ± 2.8 nmol/l) affinities. The binding of $^{125}$I-EGF is specific since it is abolished by excess unlabelled EGF in a dose dependent manner but not by excess unlabelled insulin, venom nerve growth factor, luteinising hormone, follicle stimulating hormone, growth hormone, and prolactin. Furthermore the biochemical studies revealed that the receptors were recognised by both mouse EGF and human urogastrone. Subcellular distribution of the receptors revealed that the bulk (60-70%) of the receptors were associated with the 800g heavy pellet and the rest distributed between the 15000g (mitochondrial) and the 105000g (microsomal) fractions. Binding was also found to be linear with protein concentration up to 1 mg/ml. Other conditions of the receptor assay were optimised: polyethylene glycol (PEG) precipitation and centrifugation followed by aspiration of supernatant as a method for separating specific from non specific binding was found to be superior to filtration approach. Low speed centrifugation for 30 minutes in the separation technique produced better results than shorter spinning time. Heat and trypsin pretreatment abolished the specific binding indicating the proteinaceous nature of the EGF receptor. $MgCl_2$ and dextran coated charcoal pretreatment did not improve the specific binding. Storage of prostatic tissues and homogenates at -70°C for 16 weeks did not have adverse effect on the receptor binding. Use of enzyme and enzyme inhibitors exhibited different binding characteristics: PMSF, Leupeptin and anti-trypsin improved the binding but αchymotrypsin and trypsin abolished the binding.

Comparison of receptor levels in BPH and cancer of the prostate (CaP) revealed that BPH receptor levels (mean ± SD = 125 ± 20 fmol/mg protein) were twice as high as in CaP (mean ± SD = 50 ± 11 fmol/mg protein) ($P < 0.01$). Furthermore the receptor expression in the CaP varied according to the histologic differentiation of the tumour as assessed by Gleason score (G/S): Well differentiated CaP (G/S:2-4) expressing more receptors (mean ± SD = 84 ± 13 fmol/mg protein) whilst poorly differentiated tumour (G/S:8-10) expressing little or no receptors at all (mean ± SD = 25 ± 15 fmol/mg protein) ($P < 0.01$).

The biochemical findings were confirmed by an immunocytochemical approach using a specific monoclonal antibody to the receptor sites which were localised at the basal layers of the epithelium. The ligand and antibody recognise two different binding sites on the receptor.

Evidence that EGF binding and kinase activity observed in the human prostate reside in the same protein, is provided by results obtained for affinity labelling studies which showed that EGF was bound to and increased the autophosphorylation of a macromolecule of 170000 daltons molecular weight measured by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

PGS/ABST/83 *Use this side or*

# D E D I C A T I O N

This thesis is dedicated to my mother, Madame Lucy Mami Abbey, whose foresight, faith, determination, hard work, sacrifice and encouragement provided me with the much needed foundation to make the present work possible.

And to Duro for her love, support and encouragement which provided the necessary impetus to carry on.

## ACKNOWLEDGEMENTS

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I am indebted to all my colleagues in the Department of Surgery/ Urology teaching laboratory for their helpful, constructive criticism of my work. I thank all the Urological surgeons for the constant provision of human prostate tissues. My special thanks also go to Professor A. Busuttil, head of Department of Forensic Medicine, University of Edinburgh Medical School, for his expert advice and guidance in the selection of prostate tissues for this research and also for his critical assessment of stained sections.

My grateful thanks also go to all members of staff in the Department, especially Mrs Ann Wilson for her ready assistance at all times and also for typing the abstract of this thesis.

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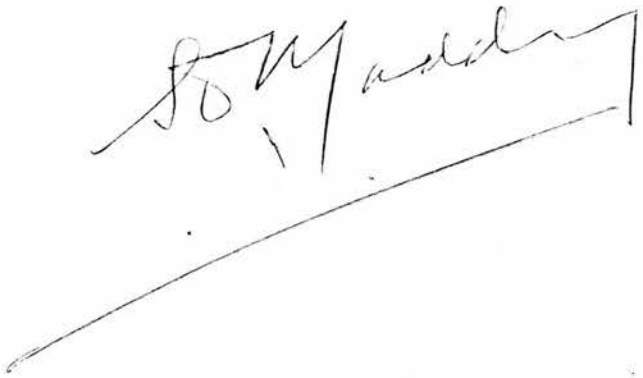
Finally, I am grateful to the Ghana Medical School for my release and especially to Professor John Pobee, the ex-Vice Dean of the Medical School, for helping to secure the WHO grant which supported the PhD programme.

Last, but by no means the least, I wish to thank Mrs Joan S. Cowper for the excellent typing of the thesis.

## DECLARATION

I, Samuel Quarcoo Maddy, hereby declare that the work embodied in this thesis is the result of my own independent investigation. This is in accordance with rule 3.4.7 of Edinburgh University Postgraduate Study Programme 1987/88.



## PUBLICATIONS

The following publications were derived from this thesis:-

1. Localisation of epidermal growth factor receptors in the human prostate by biochemical and immunocytochemical methods.

   S.Q. Maddy, G.S. Chisholm, R.A. Hawkins, F.K. Habib.
   J.Endocr. 113, 147-153 (1987)

2. Are epidermal growth factor receptors present in the human prostate?

   Fouad K. Habib and S.Q. Maddy.
   Protides of biological fluids. Vol. 35, page 315 (1987)

Both of these publications have been inserted in the Backflap of this thesis.

## ABBREVIATIONS

| | | |
|---|---|---|
| 1. | DCC | Dextran coated charcoal |
| 2. | $MgCl_2$ | Magnesium chloride |
| 3. | mEGF | Mouse epidermal growth factor |
| 4. | Uro | Urogastrone |
| 5. | EGF-R | Epidermal growth factor receptor |
| 6. | $EGF\text{-}R_1$ | Monoclonal antibody to epidermal growth factor receptor binding site on the external domain |
| 7. | Mab | Monoclonal antibody |
| 8. | PMSF | Phenylmethylsulfonyl fluoride |
| 9. | DNASE | Deoxyribonuclease |
| 10. | LH | Luteinizing hormone |
| 11. | FSH | Follicle stimulating hormone |
| 12. | GH | Growth hormone |
| 13. | PRL | Prolactin |
| 14. | v NGF | Venom nerve growth factor |
| 15. | F4 | Monoclonal antibody to the internal domain of the EGF-R |
| 16. | DSS | Disuccinimidyl suberate |
| 17. | SDS | Sodium dodecyl sulphate |
| 18. | PAGE | Polyacrylamide gel electrophoresis |
| 19. | PEG | Polyethylene glycol |
| 20. | RAMPC | Rabbit antimouse peroxidase conjugate |
| 21. | DAB | Diaminobenzidine |
| 22. | DMSO | Dimethylsulfoxide |

# CHAPTER 1

# INTRODUCTION

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## 1.1 GENERAL CONSIDERATIONS

Defective regulation of cell growth and differentiation are some of the physiological alterations which underlie the gross derangements of disease. An important goal of basic cancer biology and for that matter the biology of any other diseases is to provide molecular explanations for the defective cellular processes.

With recent developments in molecular and cellular biology, investigators have obtained penetrating insights into cell processes and their respective derangements. These are being investigated at different experimental levels including work on growth factors and their receptors.

The results of these approaches are interrelated and integrated within the framework of cell proliferation and differentiation.

The binding characteristics of epidermal growth factor (EGF) and its receptor (EGF-R) in the human hyperplastic prostate (BPH) and cancer of the prostate (CaP) is the subject of investigation described in this report.

## 1.2 THE HUMAN PROSTATE GLAND

a) The Normal Prostate: The normal prostate gland is a firm, part-glandular, part-muscular body and surrounds the beginning of the urethra. It is roughly the size and shape of a chestnut and is thought to serve a function in male fertility.

i) Location: It lies in the pelvis, behind the pubis, and in front of the rectum. Its base is directed upwards and is continuous with the bladder neck, as illustrated in Figure 1.

Figure 1. Showing the location of the prostate.

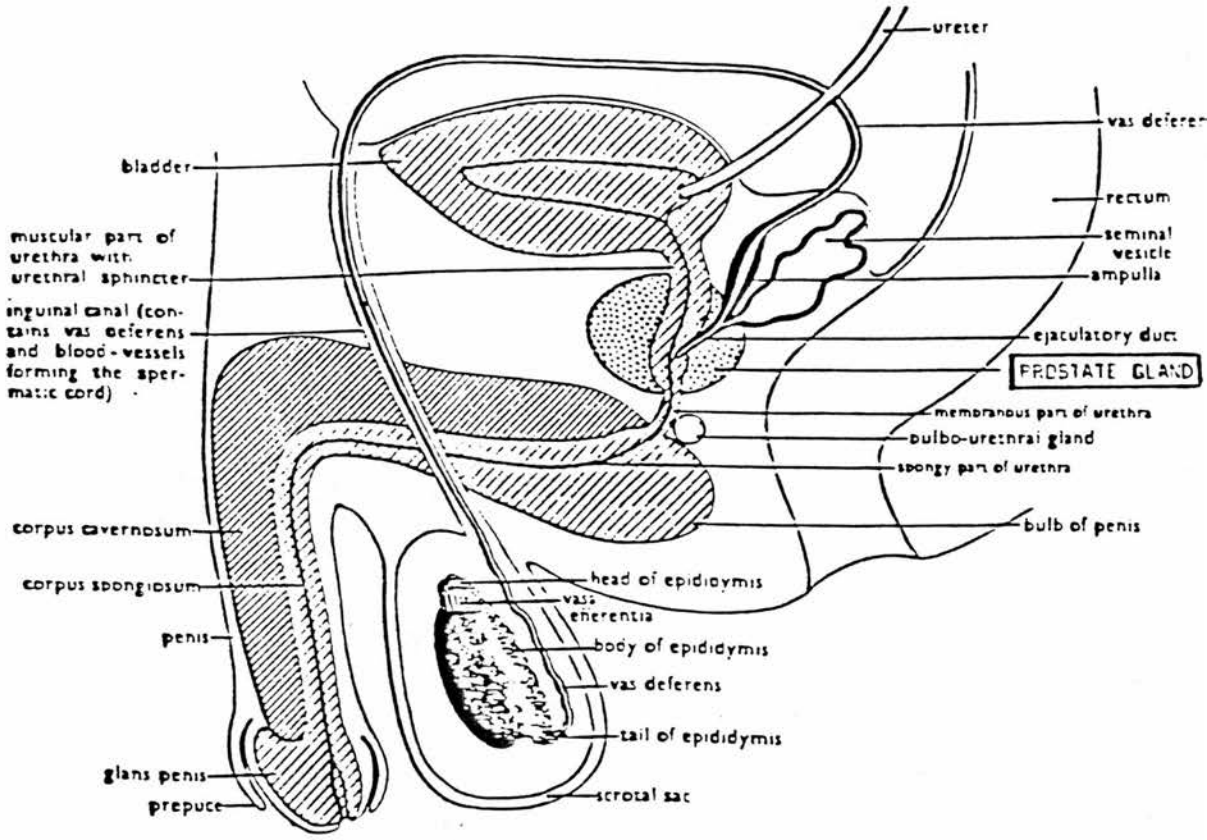

ureter
vas deferens
bladder
rectum
muscular part of urethra with urethral sphincter
seminal vesicle
ampulla
inguinal canal (contains vas deferens and blood-vessels forming the spermatic cord)
ejaculatory duct
PROSTATE GLAND
membranous part of urethra
bulbo-urethral gland
spongy part of urethra
corpus cavernosum
bulb of penis
corpus spongiosum
head of epididymis
vasa efferentia
penis
body of epididymis
vas deferens
tail of epididymis
glans penis
prepuce
scrotal sac

Figure 1.

ii) Genesis: Morphogenesis is initiated in the fetus with the appearance of solid epithelial outgrowths (prostatic buds) which grow peripherally from the urogenital sinus (prostate urethra into surrounding mesenchyme (stroma). The prostate buds become the main collecting ducts and eventually form an extensive glandular network in the stroma. (Cunha et al, 1986)

iii) Growth: The prostate gland grows slowly from birth to puberty, then rapidly to the age of 30. Thereafter it remains more or less constant in size until the age of about 45 (Swyer, 1944). From the mid-forties, prostate weight begins again to increase gradually. Berry et al (1984) correlated the size of the human prostate gland and age using data from major autopsy series (n = 740). Figure 2 is showing a graph of the mean values.

iv) Anatomy: The prostate gland consists of two sets of glands, an inner or periurethral zone, which surrounds the urethra, and an outer zone which surrounds the inner zone and is separated from the prostatic plexus of veins by a thick fibrous capsule (Figure 3). The urethra is about 20 cm long and is divided into three parts but only the prostatic part is relevant to this discussion. The prostatic part lies within the prostate gland. It is the widest part and runs vertically through the prostate. On its posterior aspect is the prostatic eminence or verumontanum, on either side of which the prostatic glands and the ejaculatory ducts open (Figure 4). The verumontanum indicates the level of the external sphincter at transurethral resections of the prostate and it must therefore be preserved.

The peripheral zone is part of the true functional prostate (McNeal, 1975) whilst the inner zone is made up of small simple

Figure 2. Illustrating the mean age adjusted size of the human prostate.

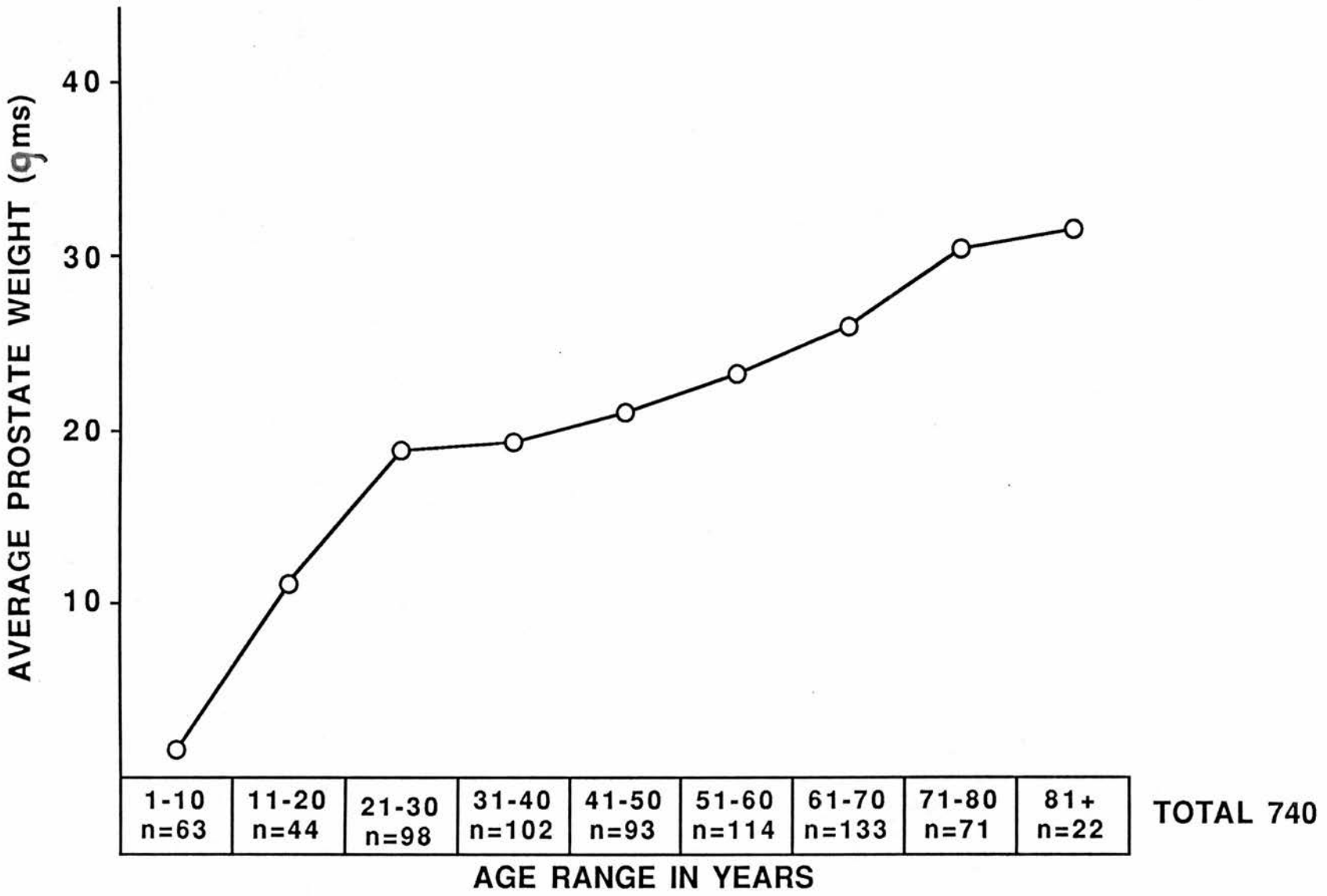
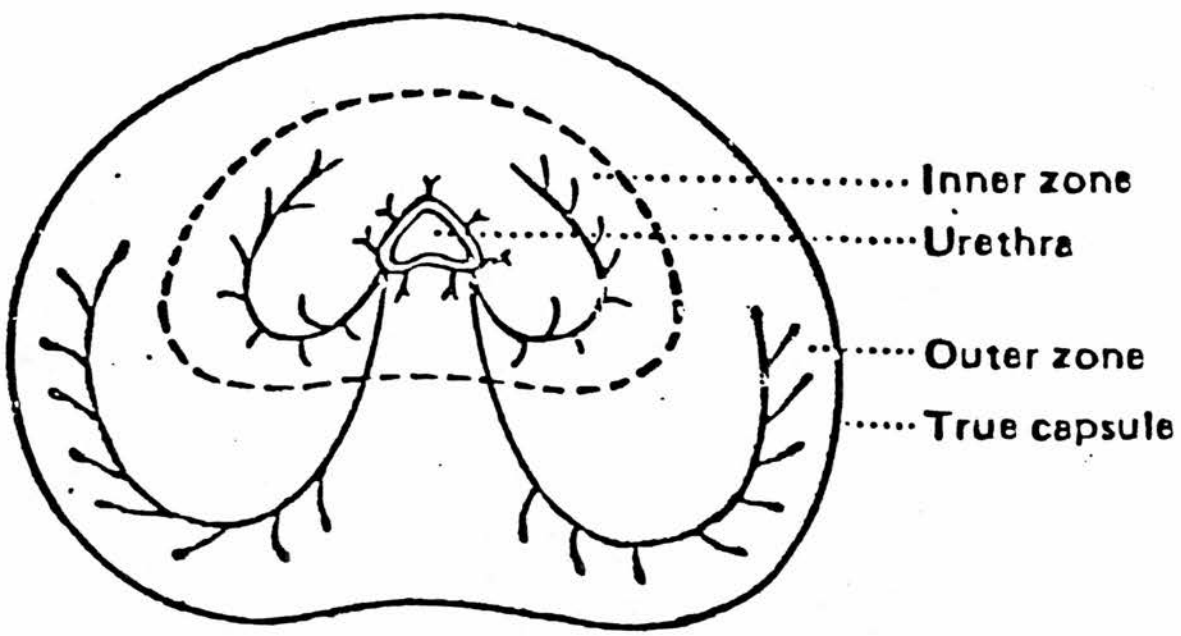

AVERAGE PROSTATE WEIGHT (gms)
40
30
20
10
1-10 n=63
11-20 n=44
21-30 n=98
31-40 n=102
41-50 n=93
51-60 n=114
61-70 n=133
71-80 n=71
81+ n=22
TOTAL 740
AGE RANGE IN YEARS

Figure 2.

Figure 3. The transverse section of the human prostate showing the concentric zones.




Transverse section of the prostate.

Figure 3.

Figure 4. Illustrating the prostatic urethra and the verumontanum.

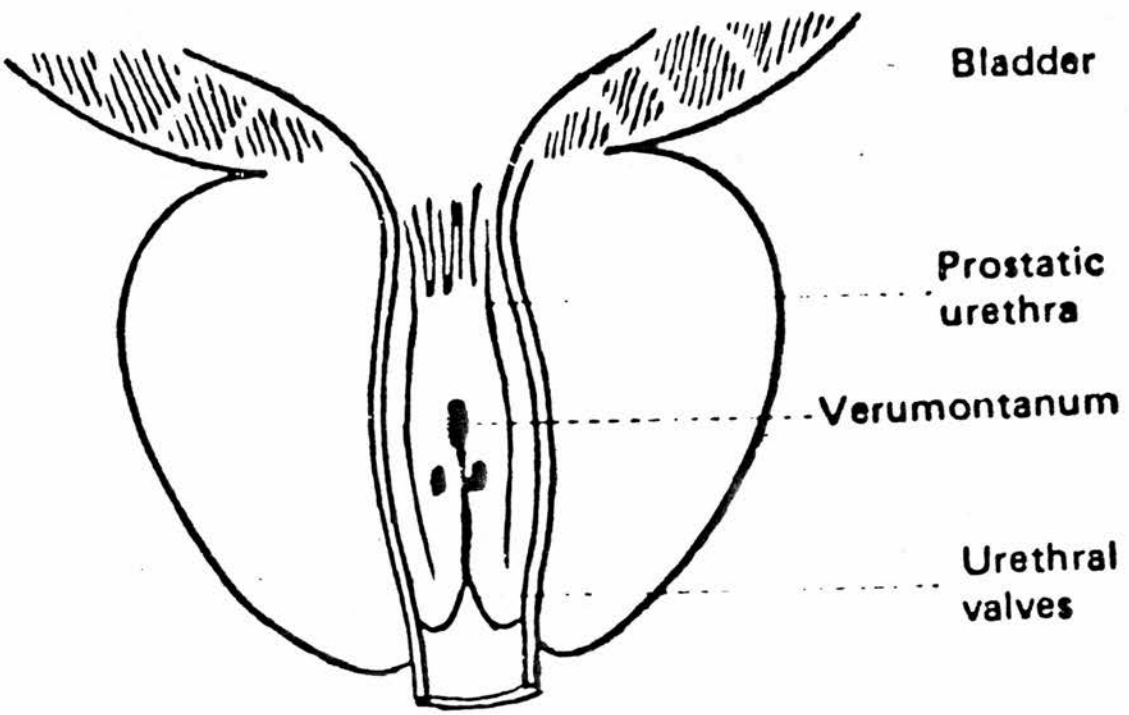



Figure 4.

structures which are not thought to contribute to the production of seminal fluid.

v) Histology: Histologically, the normal prostate is made up of small to large acinar glands and their ducts arranged in radial fashion. The prostate acini are lined with a columnar epithelium that is characteristically two cell layers thick (basal and principal cells) and peripheral ducts lined by a single layer of cuboidal epithelium that merges with the transitional epithelium of the central prostatic urethra (McNeal 1972, 1975). These glands are separated by an abundant fibromuscular stroma. The glandular and muscular elements are barely detectable in the child. They enlarge with puberty and in middle age they develop the nodules and whorls of benign prostatic hyperplasia.

vi) Function: The normal physiological function of the prostate is unknown. It was originally thought to play some role in reproductive function but the role is still not clearly defined. During emission the prostate is believed to secrete 0.5 ml of thin milky alkaline fluid containing citric acid, calcium acid phosphatase, a clotting enzyme and a profibrinolysin (Guyton, 1981). The prostatic capsule contracts simultaneously with the vas deferens so that the thin fluid of the former adds to the bulk of the semen. The alkaline characteristics of the prostatic fluid serves to neutralise the acidity of the vas deferens secretions thereby achieving an optimal pH environment for the spermatozoa (pH 6.0 - 6.5). Apart from this small contribution to reproductive function, it is thought that the prostate may be non-essential to life (Franks, 1983). It may be rather a source of more clinical problems, and also may be as

useless as the appendix vermiformis (Farnsworth, 197 ).

b) Benign Prostatic Hyperplasia (BPH): BPH is one of the most common diseases affecting 60% of men within the age group of 40 - 59 years of age and more than 95% of those over 70 years (Habitz, 1972).

i) Genesis: The aetiology of BPH is not clearly understood but it is thought that it begins in the inner or periurethral region of the prostate gland as fibrous nodules which induce the ingrowth of adjacent epithelial elements (Le Duc, 1939; Deming and Neumann, 1939). The nodules are formed presumably as a result of cellular disturbance arising entirely at the local level within its susceptible region.

ii) Presentation: The usual clinical presentation of BPH is that of urinary flow obstruction caused by bladder neck constriction. About 10% of men show clinical symptoms that merit surgical intervention and relief by transurethral resection (Robbins, 1984).

iii) Incidence: This was studied within different age groups using prostatic enlargement as the major criterion. It was found to be low in the age group below 20 year old males, whilst among octogenarians the incidence was over 50% (Randal, 1931) but using histological criteria, BPH was found to be absent in individuals below the age of 30 years, whilst over 70% of octogenarians had the condition (Moore, 1935).

Franks (1954) used classification based on "possible BPH", "microscopic BPH", "macroscopic BPH" to investigate 211 prostate specimens in relation to age. He concluded that octogenarians had 53% macroscopic, 32% microscopic and 16% possible BPH.

c) Cancer of the Prostate: In marked contrast to BPH, adenocarcinoma

of the prostate is distinctly associated with epithelial cells in the peripheral region of the prostate (McNeal, 1972; Robel, 1980). Cancer of the prostate is the commonest cancer found in the male urogenital tract (Smith et al, 1976). Mortality from prostate cancer is the fourth most common cause of death from malignant diseases in men. Approximately 4,000 fatalities occur in every year in England and Wales (Annual Report DHSS, 1974). About 6,000 new cases are also recorded in the United Kingdom every year (Tate et al, 1979). In the USA, it is the second most common cancer and the third most prevalent cause of cancer deaths for men. About 42,000 new cases are recorded every year (Klein, 1979; Silverberg, 1982). It was estimated that approximately 96,000 new cases of prostate cancer would be diagnosed in 1987 (National Institute of Health Consensus Development Conference Statement (1987)).

i) Incidence: The incidence is generally accepted as age related. The condition is rare before the age of 50. It is greatest in men between 65 and 75 years of age, although it is uncommon in those aged between 45 and 55 years.

ii) Presentation: Cancer of the prostate can remain undiagnosed and asymptomatic in aged men and be revealed only at autopsy. Conversely, it may remain dormant in younger men for many years (Griffiths et al, 1979).

iii) Aetiology: The classical work of Huggins & Hodges(1941); Huggins et al (1941) clearly demonstrated that prostatic carcinoma was androgen dependent. Objective tumour regression was obtained with castration and treatment with oestrogens. But although androgen secretions are necessary to the development of prostatic cancer, their role seems only permissive (Bouffioux, 1983). Many other

factors, e.g. genetic, infection, socioeconomic, dietetic and biochemical have also been suspected of playing a role in the occurrence of the disease (Alderson et al, 1981; Baba, 1982; Bouffioux, 1983). These observations were confirmed by Ch. Bouffioux (1984). He concluded that prostate cancer, like most malignancies, does not depend on a single aetiology but on a complex interaction, some facilitating and others inhibiting any potential oncogenic transformation. Chisholm (1980) and Chisholm and Habib (1980) have outlined in their reviews, some of the factors that may lead to prostate cancer. There is a geographic variation in age-adjusted mortality rate among people with cancer of the prostate. The rate is said to be highest in Sweden, Norway and Switzerland but lowest in the Philippines, China, Japan and Taiwan. Environmental and dietary factors may be implicated since migration from low incidence area to high incidence area increases mortality rate. Epidemiological factors have also been found to be relevant. There is a higher occurrence of cancer of the prostate among blacks in America than whites but there is low occurrence among blacks in West Africa.

Sedentary occupation, sexual activity and venereal diseases have all been associated with cancer of the prostate.

Zinc and cadmium have also been implicated in carcinogenesis of the prostate.

Kipling and Waterhouse (1967) have shown that industrial workers exposed to high levels of cadmium have a higher incidence of prostatic carcinoma. This was confirmed by Habib et al (1976) who showed that the cancerous prostate had higher levels of cadmium than BPH tissues, but found that the zinc levels in cancer of the prostate were much lower than normal counterparts. These reports

would seem to mean that high cadmium concentrations and low zinc levels in the prostate would place the prostate in a favourable condition to develop cancer. It could also be that the levels observed were the consequence of the cancer.

## 1.3 FACTORS AFFECTING PROSTATIC GROWTH IN HEALTH AND DISEASE

In order to control any disease or tumour of the prostate, it is important to understand the factors involved in growth and the mechanism by which they cause the disease. In recent years, the search for information about factors regulating the growth of the prostate has accelerated due mainly to progress made in the understanding of not only steroid biochemistry but also the implication of stromal-epithelial interaction and growth factors.

There is now a substantial volume of experimental data on the factors that may influence the growth of the gland. These factors will now be addressed:

a) Androgens: John Hunter (1786) established the relationship between the testis and the development of male sexual characteristics. In his first scientific report, he described an association between the presence of testis and the secondary sexual organs: prostate, Cowper's gland and seminal vesicles. About two and a half centuries later, Kutsher and Wolbergs (1935) reported the presence of acid phosphatase activity in the prostate gland. Gutman and Gutman (1938) established a relationship between acid phosphatase activity and the testis after observing an increase in enzyme activity during puberty when testicular function is established. This finding was confirmed by Gutman and Gutman (1939) who observed an increase in prostate acid phosphatase activity after injecting testosterone into prepubertal monkeys. These observations were later strengthened by Huggins and Hodges (1941) who demonstrated

that a decrease in serum acid phosphatase activity in prostate cancer following castration or injection with oestrogen correlated with improvement of clinical symptoms. But injection with androgens caused an increase of both acid phosphatase activity and pain (Huggins et al, 1941).

These studies on the prostate demonstrated that the growth and maintenance of the gland were largely dependent on androgen availability from the testis. The studies also laid the foundation for the endocrine treatment of prostatic carcinoma. Castration and oestrogen therapy have been the standard endocrine treatment of metastatic prostatic carcinoma. Oestrogen treatment results in the reduction of plasma testosterone levels to castrate levels, indirectly through inhibition of luteinizing hormone (LH) output from the anterior pituitary , by a negative feedback mechanism (Harper, Peeling and Cowley, 1976) or by a direct effect on the biosynthesis of testosterone in the testicular tissue (Samuels et al, 1964). But still the exact role of androgens in the prostate is not clearly defined. Since the work of Huggins et al (1941), attempts have been made to understand the actual role of testicular androgens in the growth and development of the prostate. In a number of studies describing the genesis of BPH various workers (Wojewski et al, 1965; Siiteri & Wilson, 1970; Schroedt & Foreman, 1971; Robel et al, 1971; Geller et al, 1976) tried to find out the causal correlation between androgens and development of BPH, but no evidence was presented after their investigations in in vitro culture system to show the promotion of prostatic cell proliferation by androgens. Habib et al (1979) looked at testosterone and zinc levels in BPH and cancer of the prostate. They concluded that biochemical classification of prostate tissue as BPH was only acceptable provided that dihydrotestosterone and testosterone ratio was greater than 1 (DHT : T > 1). This would seem

to support direct androgen involvement in BPH development. It is also consistent with the views of other workers (Webber, 1980; Syms et al, 1982; Sestili et al, 1983; Syms et al, 1985) who reported on direct androgen stimulation. But recently McKeehan et al (1984) thought that there was no direct androgen stimulation. They showed that proliferation of the normal prostate epithelium in serum-free media was enhanced by protein hormones and growth factors but not by androgens. But very recently the growth rate and EGF receptor activity of human prostate tumour cell line LN CaP has been stimulated by androgens (Shuurmans et al, 1988). In earlier reports Brehmer et al (1972) also indicated that androgens inhibited fibroblastic cell proliferation.

The confusion regarding the status of androgens in the prostate indicates that androgens per se are not able to cause BPH and CaP. Their action must therefore be mediated via other factors. The interaction between the stroma and the epithelium in the prostate has been shown to be important.

One distinct feature of the human prostate is the capacity to metabolise testosterone to a variety of metabolites of which dihydrotestosterone is biologically the most important. The conversion of testosterone to dihydrotestosterone is now believed to be an obligatory step in the mediation of androgen action in the human prostate. The widely accepted two step model for androgen action in the prostate is as follows: Testosterone is converted in the cytoplasm into the more potent androgen dihydrotestosterone (DHT) by membrane bound enzymes 5$\alpha$ reductase. The DHT then binds to the cytoplasmic androgen receptor and the receptor DHT complex is translocated into the nucleus (King & Mainwaring, 1974; Mainwaring, 1977; Jensen et al, 1982). This model is in the process

of modification. The demonstration that oestradiol receptor is localised in the nucleus in the rat uterus and other tissues (King et al, 1984) has led to the suggestion that the androgen receptor is similarly restricted. This speculation was confirmed by Houston et al (1985) who looked at 5∝reductase activities in human prostatic nuclei and microsomes and concluded that the human prostatic tissue contains only one form of 5∝reductase which is located exclusively in the nucleus. This finding is having important implications for the mechanism of steroid action in the prostate. The new mechanism would seem to be based on a one step model and not a two step model as proposed. Regardless of the initial localisation of the receptor, it is the binding of the nuclear steroid receptor complex to the acceptor site in the prostatic chromatin possibly associated with the nuclear matrix (Wang et al, 1984; Colvard and Wilson, 1984) that is assumed to initiate specific gene transcription and/or replication.

b) Stromal Epithelial Interaction: From histological analysis, the prostate is now known to be composed of both stromal and epithelial elements. This knowledge has helped to elucidate the role of androgens in the prostate and has extended the mechanism of action to encompass stromal epithelial interaction. Findings of several investigators have emphasised the importance of the stroma in the control of prostatic growth development, maintenance and disease. Cowan et al (1977) demonstrated that although testosterone was metabolised in both the stroma and epithelium, the majority of the 5∝reductase activity resided in the stromal fraction. Subsequent studies also showed that androgen receptor levels in the stromal-epithelial compartments were not the same (Lahtonen et al, 1982). These results suggest that the prostatic stroma plays an important role

both structurally and biochemically in maintaining the normal function of the prostate. Franks et al (1970) showed that epithelial cells derived from hyperplastic prostate did not grow well in culture in absence of stroma. Cunha (1972; 1973), Cunha et al (1980); Lasnitzki and Mizuno (1980) and Cunha (1984) also established the importance of the stroma in the development of the normal morphology of the prostate and in normal function of the epithelial cells within the gland. By tissue recombinant experiments consisting of murine epithelium and mesenchyme from the embryonic urogenital sinus of normal and testicular feminised mice, these workers demonstrated that the mesenchyme was responsible for the androgen induced development of the tissue. At this early stage of development of the prostate, Shannon and Cunha (1983; 1984) showed that 5$\alpha$ DHT receptors were already present in the mesenchyme but were undetected in the epithelial cells. These results confirmed that the stroma was in control of the gland's growth and development. But other workers had previously established the presence of androgen receptors in the prostate epithelium (Coffey, 1974; Touchimaa and Niemi, 1974; Bruchovsky et al, 1975; Stumpf and Sar, 1976; Liao, 1977; Krieg et al, 1981). Administration of androgens to castrated males elicited growth of the prostate and specifically stimulated epithelial proliferation as judged by analysis of $^{3}H$ thymidine labelling index and mitotic index of the epithelium. Although the presence of androgen receptors has been established in both the stroma and the epithelium, the evidence from the literature suggest that the bulk of the receptors reside in the stromal compartment (Franks et al, 1970; Cowan et al, 1977; Krieg et al, 1981; Lahtonen et al, 1982; Shannon and Cunha, 1983; 1984). This follows that the biological action of androgen is achieved by complexing with its nuclear receptor in the stroma (Cunha, 1984; Wang et al, 1984;

Colvard and Wilson, 1984; Houston et al, 1985). The result of the complexing is the synthesis of specific growth factor or factors which then proliferate the epithelial cells (McKeehan et al, 1984; Smith et al, 1985).

It has been suggested that BPH is basically a stromal disease which is made up of nodules consisting of a mixture of stromal and epithelial elements (Franks, 1976). Furthermore, it has been shown that in BPH, there is an increase in fibrostromal volume over the volume of glandular elements in the normal gland of young males (Bartsch et al, 1979).

c) Growth Factors: Growth is accompanied by change that results from either an increase in cell size (hypertrophism) or cell number (hyperplasticity). Various agents are involved in the growth regulatory process. One group of such agents is referred to as growth factors. They are molecules which have emerged as a major subset of hormones that will probably far eclipse the more classical endocrine polypeptide hormones. Polypeptide growth factors differ from classical hormones by the method of transport to target cells. Endocrine substances are transported to distantly removed target cells, by means of the blood stream. Paracrine agents differ by the fact that the released hormone or growth factor travels to its target cell by diffusion. A third class, autocrine interaction, described by Sporn and Todaro (1980) differ from paracrine in that the target cell is the same as that actually producing the growth factor. The three basic models are illustrated in Figure 5. But the consensus of opinion is that the majority of polypeptide growth factors act in a paracrine model (James and Bradshaw, 1984).

i) Mechanism of action: A clear picture of the mechanism of action of any polypeptide growth factor is not available.

Figure 5. Illustrating schematically, the endocrine, paracrine and autocrine actions.

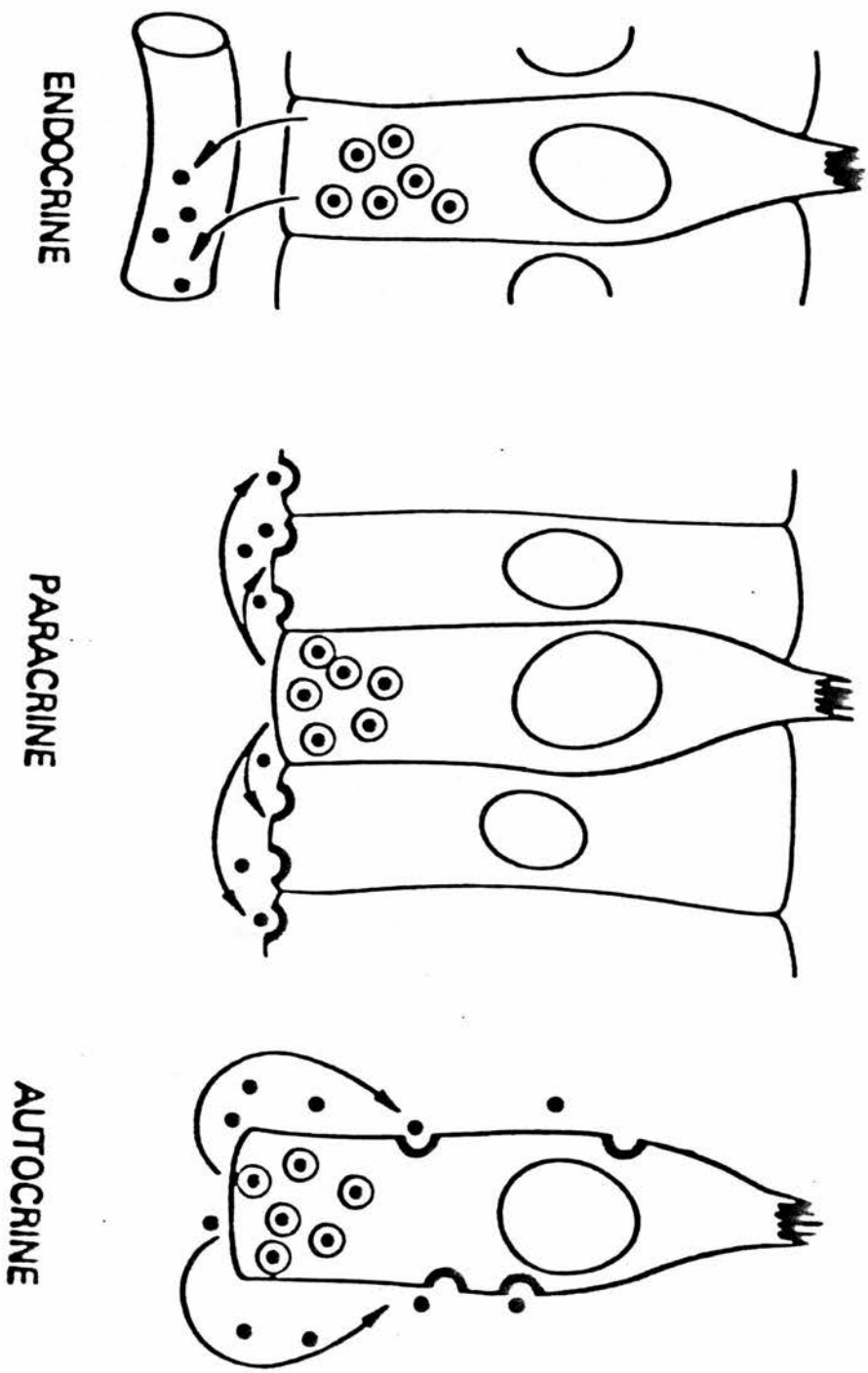



Figure 5.

However, certain general characteristics common to the activity of all polypeptide growth factors seem evident and these include: initiation of the response by formation of a specific high affinity cell surface receptor complex; generation of immediate signal that is responsible for at least a part of the overall mechanism; internalisation of both the ligand and its receptor, largely through the mediation of clathrin-coated pits by endocytosis; and the ultimate degradation of both the receptor and ligand by the action of lysosomal enzymes. Although information continues to accrue regarding processes and events that are stimulated by the interaction of growth factors with their responsive cells, yet the basic signals that initiate long and short term effects are still not identified. For example, the importance of internalisation of growth factor receptor complex by endocytosis and the role of intracellular receptors as sites for further activity or as messengers themselves, are not clearly defined (James and Bradshaw, 1984).

ii) Growth Factor and Androgen Action: The view that growth factors are the actual growth regulators in the prostate has been supported by many workers. McKeehan et al (1984) have shown that proliferation of rat prostate epithelial cell populations was enhanced by protein hormones and growth factors but not by androgens. Furthermore, the mitogenic effects of prolactin and EGF on the rat prostate tumour have been published (Smith et al, 1985). Tenniswood (1986) in an hypothesis strengthened the work of Cunha et al (1986) by highlighting the implication of growth factors in the mechanism of androgen action.

The nature of the growth factor or factors implicated is not

known. This has therefore engaged the attention of many biochemists and molecular biologists.

## 1.4 EPIDERMAL GROWTH FACTOR (EGF)

The literature has described a whole range of growth factors and their receptors. But EGF is one of the best characterised mitogens for the study of mammalian cell proliferation and/or differentiation. Furthermore, its importance to biochemical research stems from the fact that it has a potential role in human disease (Carpenter et al, 1978) EGF was therefore selected for this investigation.

a) Characteristics of EGF: EGF was discovered when fractions from submaxillary glands not containing nerve growth factor (NGF) were shown to induce premature eyelid opening and tooth eruption in the new born mice (Cohen, 1962). EGF was soon isolated (Cohen and Elliot, 1963) and its amino acid sequence determined (Savage et al, 1972). EGF isolated from human urine was found to be identical to B- Urogastrone, a polypeptide inhibitor of gastric acid secretion (Carpenter and Cohen, 1975; Gregory, 1975). EGF is mitogenic for a variety of mesenchymal cells and epithelial cells in culture (Carpenter and Cohen, 1975). EGF is a 6KDa polypeptide consisting of a single chain of 53 amino acids with intrachain disulphide bonds (Taylor et al, 1972). But a large protein precursor of 976 - 1217 amino acids was provided by Gray et al (1983); Scott et al, 1983); Carpenter and Cohen (1984). They used the nucleotide sequence of complete CDNA derived from a male mouse submaxillary gland DNA. The molecular weight of the precursor was about 130,000 dalton. Within this prepro EGF molecule were 7 EGF-like peptides.

At tissue and cellular levels EGF stimulates protein, RNA and DNA synthesis (Carpenter and Cohen, 1979; Das, 1982; King and

Carpenter, 1983). It also increases uptake of nutrients including $Ca^{++}$ (Sawyer and Cohen, 1981) and alters phospholipid metabolism including phosphoinosityl (PI) turnover (Sawyer and Cohen, 1981; Smith et al, 1983) and increases release of arachidonic acid (Shupnik and Tashjian, 1982; Aoyagi et al, 1985).

In most tissues, there are two major classes of cell membrane linked receptor systems which control cell proliferation. The best characterised is mediated by production of cyclic AMP from adenylcyclase which become activated by different mitogens. The less well characterised system in which EGF is involved activates PI turnover, $Ca^{++}$ mobilisation, cyclic GMP production and the release of arachidonic acid (Aoyagi et al, 1985). This establishes a link between EGF, PI and cell proliferation (Michell, 1984).

b) EGF Receptor: The biological action of EGF is thought to be mediated by the interaction with its specific high affinity cell surface receptors with apparent dissociation constant (Kd) of $10^{-9}$ - $10^{-10}$ M (Hollenberg and Cuatrecasas, 1973; Carpenter and Cohen, 1975, 1979; Hollenberg et al, 1979; Taketani and Oka, 1982).

The cell receptor for EGF is the best understood growth factor receptor and has served as a paradigm for other growth factor receptors. The receptor was first purified from A431 cells, a cell line derived from a human squamous carcinoma which has a large number of EGF receptors (Fabricant et al, 1977; Cohen et al, 1982; Kawamoto et al, 1983). The receptor is an integral 150 - 170 KDa membrane protein exhibiting an extracellular binding domain that serves to bind the ligand EGF, a transmembrane region and intracellular domain facing the cytoplasm. The cytoplasmic domain exhibits the tyrosine Kinase function and presumably binding sites for ATP phosphorylation substrates (Cohen et al, 1980, 1982). In response to EGF the receptor is capable of

autophosphorylation on tyrosine residues (Dailey et al, 1978; Gill and Lasar, 1981). The endogenous kinase mediates tyrosine phosphorylation but other kinases are thought to be responsible for phosphorylation of serine and threonine. Figure 6 is showing a schematic representation of the EGF receptor. EGF binding sites appear to be functionally heterogeneous because a small portion of the EGF receptor binds ligand with high affinity and a large portion binds with low affinity (Kawamoto et al, 1983; Gregoriou and Rees, 1984). It is believed that the small number of high affinity receptors are involved in the generation and maintenance of the mitogenic signals. The function of the low affinity receptors is not clear but it is now known to be inhibitor of differentiation (Boonstra et al, 1985). Binding of EGF to the receptor leads to a decrease in the receptor density on cell surface (down regulation), as the growth factor receptor complex is internalised into the receptosomes pending subsequent degradation which precedes the generation of the mitogenic signal (Pastan + Willingham, 1981; Cohen, 1987). But a recent evidence indicates that the EGF receptor complex is not degraded but recycled back to the surface of the membrane (Teslenko et al, 1987).

c) The Truncated Receptor: The oncogene v-erb B of the avian erythroblastosis virus (AEV) codes for a product homologous to a portion of the EGF receptor. There is a striking homology between EGF receptor and the deduced amino acid sequence of the v-erb B oncogene product (Downward et al, 1984). The chief difference observed is the absence of EGF binding domain in the retroviral version. This remarkable finding established that the receptor protein itself has the potential for transformation. A tyrosine kinase activity was demonstrated for the v-erb B gene (Decker, 1985; Gilmore et al, 1985). The v-erb B protein is truncated for the

extracellular ligand binding domain of the EGF receptor but the shortened cytoplasmic domain retains the protein kinase site (Yamamoto et al, 1983). When activated by ligand the EGF receptor is internalised whereas the v-erb B product in the absence of ligand binding site remains at the cell surface. The kinase activity associated with the v-erb B protein is constitutively expressed to a "turned on" state regardless of the growth factor. The v-erb B protein must reach the plasma membrane to be transforming (Schmidt et al, 1985). Figure 6 is also showing the schematic representation of the truncated EGF receptor. Oncogenes like v-erb B are capable of causing carcinogenesis, because deletion of the gene from the viral genome renders the virus incapable of causing cancer. 30% of transforming proteins known, including v-erb B oncogene product, have tyrosyl kinase activity (Gill and Hasar 1981; Hunter and Cooper, 1981). In non-transformed cells phosphotyrosine represents less than 0.03% of total phospho-amino acids, but in transformed cells this level is increased approximately 30-fold (Sefton et al, 1980). Structurally, the cytoplasmic portion of the EGF receptor has an average of 25% homology to the kinase domain of several transformed proteins, but 95% homology has been observed with v-erb B product.

The identification of growth factors, oncogenes and their products is shedding new light on the involvement of polypeptides in carcinogenesis. The mechanisms of oncogene activation are not fully understood. This has therefore opened a way for cellular exploration at the molecular level.

d) EGF in Carcinogenesis: Cancer cells appear able to take on properties which allow them to escape from the controlling mechanisms of their tissues of origin and to survive, independent of that tissue.

Figure 6. Illustrating schematic representation of the EGF receptor and the truncated version of the receptor.

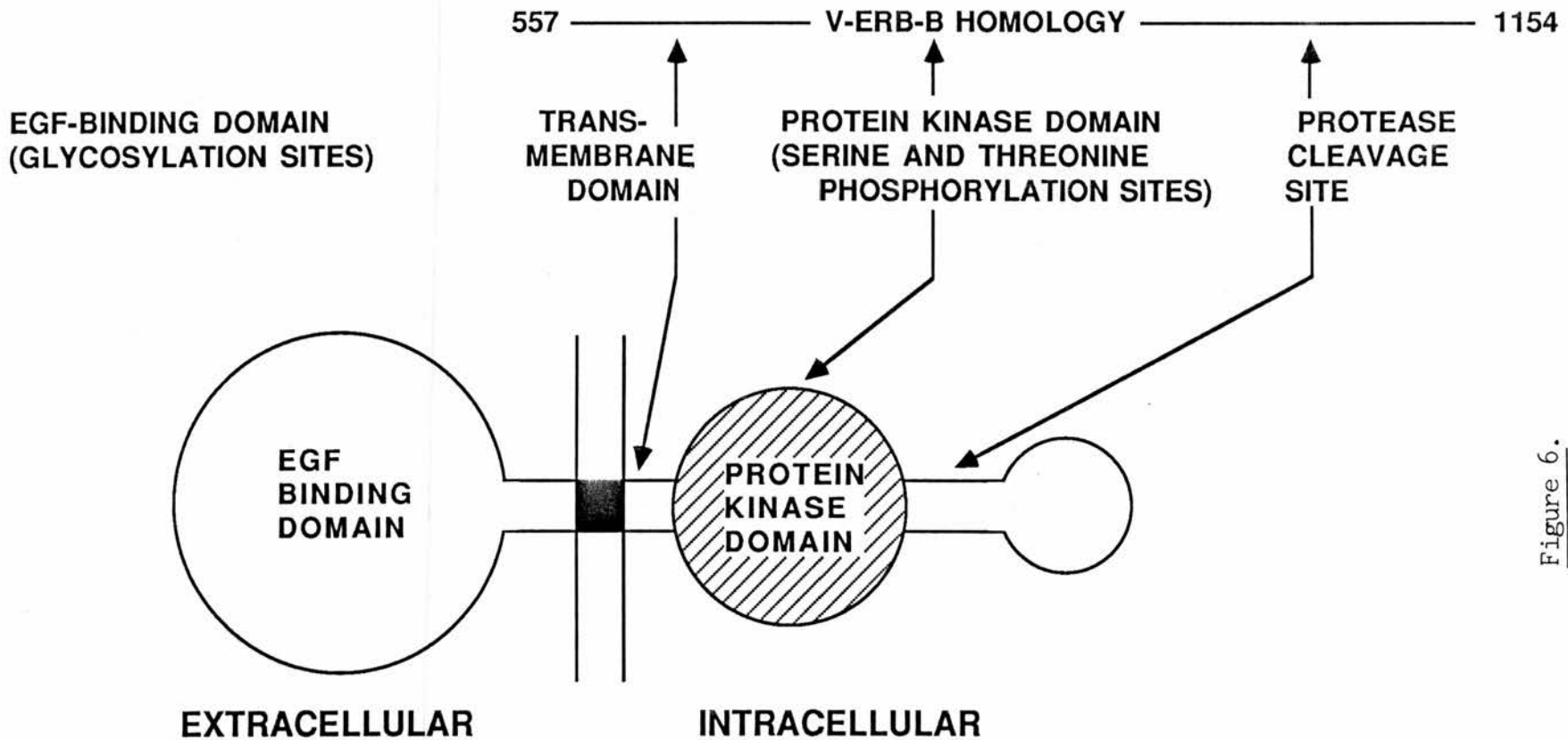

557
V-ERB-B HOMOLOGY
1154
EGF-BINDING DOMAIN
(GLYCOSYLATION SITES)
TRANS-
MEMBRANE
DOMAIN
PROTEIN KINASE DOMAIN
(SERINE AND THREONINE
PHOSPHORYLATION SITES)
PROTEASE
CLEAVAGE
SITE
EGF
BINDING
DOMAIN
PROTEIN
KINASE
DOMAIN
EXTRACELLULAR
INTRACELLULAR

Figure 6.

Many lines of evidence have been produced to suggest that EGF is an important growth factor implicated in the transformation and proliferation of some neoplasms. For example, when EGF was added to normal cells, it elicited certain responses which are associated with neoplasia.

Many of these effects were reversed upon the removal of EGF (Carpenter and Cohen, 1975; Westermark, 1976; Kirkland et al, 1979). Furthermore, EGF has been found to enhance the carcinogenic effect of methylcholanthrene in skin (Reynolds et al, 1965; Rose et al, 1976). Some transformed cells produce transforming growth factor alpha (TGF$\alpha$) which interact with EGF receptors to stimulate EGF receptor autophosphorylation and achieve all the biological effects identical to EGF (Todaro et al, 1980; Roberts et al, 1982; Anzano et al, 1983; Carpenter et al, 1983). TGF$\alpha$ will be described in detail under Chapter 1.4 e (ii). EGF has been shown to suppress the immune system. Immunosuppression is usually considered to permit cancer growth rather than cause it. Injection of EGF one day before sheep red blood cell (RBC) administration suppressed production of antibodies to the antigen. It is believed that IgG response is more affected than IgM response. IgG is more T cell dependent than IgM. Helper T cell is suggested to be the target of EGF induced immuno suppression. Furthermore, delayed hypersensitivity response to 2, 4 dinitro fluorobenzene is said to be caused by EGF. Salivary gland extract which contains EGF (Cohen, 1962) has been observed to cause atrophy of lymph nodes and the thymus (Roberts et al, 1976). Finally, EGF enhances viral transformation of cells. It produces effects similar to those produced by 12 - O tetradecanoylphorbol - 13 - acetate (TPA), a classic tumour promoter (Fisher et al, 1979).

e) Interaction of EGF, Protein Kinase C and Tumour Promoters

EGF may help to regulate its endogenous tyrosine kinase by promoting $Ca^{++}$ influx, phosphoinositol (PI) turnover, protein kinase C activation and phosphorylation of receptor tyrosine kinase (Shupnik and Tashjian, 1982).

Tumour promoters such as tetradecanoylphorbol - 13 - acetate (TPA) produce their effects on EGF receptor by their almost irreversible activation of protein kinase C and decreasing its need for $Ca^{++}$ and diacylglycerol (DG) (Nishizuka, 1984; Cochet et al, 1984; Friedman et al, 1984).

TPA stimulates phosphorylation of the EGF receptor in the same sites as purified protein kinase C (Hunter, 1984; Cochet et al, 1984; Iwashita and Fox, 1984). Therefore, protein kinase C was found to be the major binding site for TPA and other active phorbol esters as well as mediating the actions of other chemically distinct tumour promoter (Nishizuka, 1984; Friedman et al, 1984).

The consequence of either TPA or protein kinase C phosphorylating the EGF receptor appears to be a decrease in its ability to autophosphorylate and inhibit EGF binding (King and Carpenter, 1983).

f) Other Growth Factors in Carcinogenesis: There are other growth factors implicated in carcinogenesis, which transform cells through different mechanisms. Two of such growth factors are (i) Platelet derived growth factor (PDGF) and (ii) Transforming growth factor $\alpha$ (TGF$\alpha$):-

i) Platelet derived growth factor (PDGF): This is a major mitogen in serum. It is synthesised in megakaryocytes (Chernoff et al, 1980) packaged into platelets in granules and released from platelets activated by thrombin or at sites of blood vessel

injury (Witte et al, 1978; Kaplan et al, 1979). PDGF is thought to bind to and initiate its biological function on cellular substrates of the blood vessel wall. PDGF dimer is composed of a 14 - 18 KDa A chain disulphide bonded to a 16 KDa B chain (Johnson et al, 1982). B chain is defined as the product of the "c-cis" gene (Johnson et al, 1984). The B chain was found to have > 90% homology with the protein product of the "v-cis" oncogene P28 "v-cis" (Waterfield et al, 1983). P28 "v-cis" is the transforming protein of the simian sarcoma virus (SSV), an acute transforming virus. The "v-cis" oncogene is responsible for transforming and maintaining the transformed state of SSV transformed cells. The protein encoded by "c-cis" gene is shown to be nearly identical immunologically to PDGF.

ii) Transforming Growth Factor $\alpha$ (TGF$\alpha$): This was identified when it was observed that transformed cell lines had reduced numbers of binding sites for EGF (Todaro et al, 1976; Salomon et al, 1987). It was later found to be due to an activity secreted by the transformed cells, termed sarcoma growth factor (after those cells had been transformed by murine sarcoma virus.) (Delarco and Todaro, 1978; Anzano et al, 1983). The activity was later isolated and is now known as TGF$\alpha$. It has a mol.wt. of 5.6 KDa. Amino acid sequence showed that it is a single chain polypeptide of 50 amino acids with three disulphide bonds in homologous positions (Derynck et al, 1984). TGF$\alpha$ and EGF have been shown to directly compete for binding to the EGF receptor despite the 50% homology between EGF and TGF$\alpha$ (Derynck et al, 1984).

TGF$\alpha$ produces all the physiological responses expected of EGF including precocious eyelid opening in new born mice (Smith

et al, 1985). TGFα has extensive sequence homomogy with EGF and binds to EGF receptor. TGFα accelerates epidermal regeneration and healing of second degree burns. It has not so far been demonstrated in non-neoplastic adult tissues and may represent the embryonic form of EGF that is inappropriately expressed in neoplastic cells.

g) EGF Receptor Levels in Normal and Cancer Cells: The development of monoclonal antibody specific to the EGF receptor binding sites (Waterfield et al, 1982) allowed many investigators to combine the techiques of radioligand assay (Carpenter and Cohen, 1975; 1979) and immunocytochemistry (Guesdon et al, 1979; Delellis et al, 1979; Van Noorden and Polak, 1983) to measure specifically, the receptors for EGF in many normal and cancerous cells and tissues.

EGF receptor levels vary according to the cell type, the stage of differentiation of the cell and the presence of other factors.

The receptor expression in cancer is higher than in normal counterparts. Hendler+Ozanne (1984), Cowley et al (1986) observed higher EGF receptor levels in human squamous cell lung cancers and squamous carcinoma cell lines respectively than in normal cells. Furthermore, some well differentiated tumours express higher receptor levels, whilst poorly differentiated cancers express little or no receptors. Libermann et al (1985), Ozanne et al (1985), Bradley et al (1986) observed higher receptor levels in human glioblastomas, epidermoid malignancies and human colon carcinoma cells respectively than in poorly-differentiated counterparts. Overexpression of the EGF receptors has been associated with gene amplification and rearrangement (Libermann et al, 1985), gene translocation, amplification and rearrangement (Ozanne et al, 1985). Furthermore, the presence of retinoic acid (Dicker and Rozengurt, 1979;

Rees et al, 1979; Adamson and Rees, 1981) and glucocorticoid (Baker et al, 1978) also increases EGF receptor levels. The influence of the cell cycles on receptor level has been demonstrated (Robbinson et al, 1982). Loss of receptors in the poorly differentiated cancers is mostly due to production of TGF (Todaro et al, 1980; Salomon et al, 1987), synthesis of v-erb B oncogene product (Downward et al, 1984) or hormonal implication (Jia et al, 1985; Zachary and Rozengurt, 1985; Traish et al, 1987). Furthermore, chemical transformation (Lee Weinstein, 1978; Robbinson et al, 19 ; King, 1985) and cell differentiation (Gospodarowicz et al, 1978) have been shown to cause loss of EGF receptors. Furthermore, receptor gene loss is also implicated (Gamou et al, 1987). The development of the monoclonal antibody to the internal domain of the EGF receptor allowed other workers (Beguinot et al, 1985; Gullick et al, 1986; Berger et al, 1987) to search for the v-erb B oncogene product in the poorly differentiated cancers. But these reports revealed absence of the truncated receptors. Other reasons for loss of receptors are at present unclear. In other tumours, the poorly-differentiated tissues expressed more EGF receptors (Neal et al, 1985; Sainsbury et al, 1985; Yasui et al, 1988) than other histological grades of the cancer. Whilst in some tissues, although an increase in receptor level was observed in the cancer compared with normal, there was no change in the receptor levels in the histological grades (Sakai et al, 1986; Hwang et al, 1986).

The presence of EGF receptors was also sought through the use of crosslinking reagents which labelled the EGF receptor with $^{125}I$ - labelled ligand, prior to SDS - PAGE electrophoresis. The purpose of this was to demonstrate the molecular weight of the

receptor which was usually in the range of molecular weight 150 - 170 KDa (Mukku + Stancel, 1985). To further understand the receptor function, tyrosine specific kinase activity and other kinase activities forming the inherent part of the receptor molecule were identified through phosphorylation experiments (Mukku + Stancel, 1985). EGF is known to stimulate phosphorylation of substrates (Cohen et al, 1980; King and Gates, 1985). The literature is full of information on EGF receptor expression in normal and cancerous cells and tissues. The general picture is that the receptor expression is low in normal cells and tissues. But in cancer the pattern of receptor expression varies according to the type of cell or tissue and the grade of differentiation, some tissues expressing more in the well differentiated than the poorly differentiated, whilst in other tissues the reverse is observed. But there is no such information available on the human prostatic tissue.

h) Association of EGF and other growth factors with the prostate:

Studies on growth factors associated with humans date back to the late 1930s when it was observed that human urine contained a potent inhibitor of gastric acid secretion (J Gray et al, 1939) and possibly other antiulcer factors (Sandweiss et al, 1941).

However, Cohen (1962) isolated EGF from the submaxillary gland of the mouse (Chapter 1.4), which Gregory (1975), Hollenberg and Gregory (1976) showed was identical to urogastrone isolated from human urine. The view that growth factors are the actual growth regulators of the prostate led many workers to direct their attention to identify the nature of the growth factor in the prostate. Identity was sought through detection of the ligand or the receptor in the prostate tissue, but most of the earlier investigators concentrated on the detection of EGF in the gland. Elder et al (1978) were the

first to investigate the human prostate for the presence of urogastrone EGF using antiserum to the ligand in an immunocytochemical technique, but this revealed the absence of fluorescence in the prostate with antiserum to the ligand. On the other hand, Hirata and Orth (1979) using radioimmunoassay technique, measured less than 1 ng EGF in the prostate obtained from a 48 year old man at autopsy. This was the first demonstration of EGF in the human prostate. In the meantime, Jacobs and Lawson (1980) demonstrated the presence of a growth promoting factor in crude extracts of human prostate tissue prepared from BPH, well differentiated adenocarcinoma and postpubertal prostate. This was based on the original idea of Deming and Neumann (1939) who suggested that a growth promoting substance produced by the prostate might be the cause of BPH. Story et al (1983) undertook to compare the BPH crude extract with EGF but after a number of studies involving the interaction of the BPH extract and EGF with human cultured fibroblast, they concluded that EGF and the extract did not recognise the same receptor, and that the major growth factor activity of BPH extract was not due to EGF. This report, coupled with the findings of Hirata and Orth (1979) showed that there were more than one growth factors present in the human prostate. It is also in line with the recent suggestion that multiple growth factors were required to provide the maximum stimulation of specific cell type and to provide the fine tuning necessary for co-ordinated development, and to maintain tissues in adult state (Walthall and [illegible] 1981; Leof et al, 1982; Sporn and Roberts, 1988). See also Chapter 1.3 (cii).

The mechanisms by which the fine tuning effect is achieved by the presence of multiple growth factors is not understood, but it is suggested that the presence of one growth factor may modulate the mitogenic activity of another growth factor. Transforming growth

factor β stimulates the growth of certain fibroblasts in vitro in the presence of PDGF but inhibits this effect in the presence of EGF (Roberts et al, 1985). Similarly, PDGF decreases the affinity of EGF receptor for its ligand (Bowen-Pope et al, 1983; Zachary and Rozengurt, 1985). In this regard, whether the activity of the BPH extract is PDGF-like substance or not, is yet to be determined. This was undertaken by Jinno et al (1986), who investigated the biochemical and physico-chemical properties of the BPH extract. This revealed that it has a multi-molecular form comprising three active components (80,000, 43,000 and 10,000 daltons). It is acidic and susceptible to heat and trypsin, and is devoid of esteropeptidase. These properties make the BPH extract different from other growth factors like EGF, PDGF, TGF and Fibroblast growth factor. For example, EGF is heat resistant even to 100°C for 10 minutes and has esteropeptidase. PDGF and FGF are basic. The characterisation makes the prostate growth factor (PGF) a separate growth factor, probably working in concert with other growth factors in the prostate. Examination of the activity according to prostatic disease revealed that there was no difference between the levels of activity in BPH and CaP, but there was a big difference ($P<0.001$) between levels in normal and BPH as well as CaP, BPH and CaP having more activity than normal prostate. In the meantime, other workers searched for the growth factor in prostates of other experimental models. Shikata et al (1984) using immunocytochemical technique involving antiserum to EGF, demonstrated the presence of the growth factor by observing fluorescence in the guinea pig prostate with antiserum to the ligand. Recently, Traish+Wotiz (1987) using radioimmunoassay method based on the commercial EGF RRA Kit (Biomedical Technologies, Stoughton MA), were able to demonstrate the presence of measurable amounts of EGF or

EGF-like substance (5 - 10 ng/g tissue) in the prostate homogenate from intact rats.

Furthermore, prostate derived growth factor (PrDGF) obtained from the rat prostate was purified and partially characterised (Maehama et al, 1986). When PrDGF was compared with other known growth factors (EGF, PDGF, FGF, TGF$\alpha$ and TGF$\beta$) in chemical composition and biological properties, it was found to be different, suggesting that PrDGF had not been previously described, but it has a lot in common with TGF $\beta$ with regard to molecular weight, presence of disulphide bonds, and stability in heat and acid. Whether PrDGF is truly different from TGF $\beta$ requires further work to establish this fact. However, PrDGF appears to be a growth promoting activity with the potential to mediate some of the activities related to prostatic growth, development and perhaps hyperplasia and neoplasia, but the search for EGF in the human prostate had not been abandoned. Recently Gregory et al (1986), using immunocytochemical method involving antiserum to urogastrone EGF, made another attempt, after Elder et al (1978) to localise EGF in the human prostate but again this revealed the absence of the ligand in the human prostate.

This report is interesting. The immunocytochemical technique using identical antiserum produced positive result in the guinea pig prostate but the same technique failed to show evidence of the presence of the ligand in the human prostate and this happened on two separate occasions. This may be due to the low quantity of EGF in the human prostate (less than 1ng/g) (Hirata and Orth, 1979) compared to the large quantity in the rat prostate (5 - 10 ng EGF/g) (Traish + Wotiz 1987). It is therefore reasonable to speculate that the positive immunocytochemical result obtained in the guinea pig prostate was probably due to the presence of a much higher quantity of EGF in the

guinea pig prostate than the human's. The low level in the human prostate might be below the detection limit of the technique. Secondly, the fact that using radioimmunoassay technique, it was possible to measure less than lng EGF/g showed that radioimmunoassay technique was more sensitive to be able to pick smaller quantities of the ligand than the immunocytochemical method would be capable of.

Gregory et al (1986) then investigated the human prostate for the presence of urogastrone EGF receptor using radio ligand assay technique as described by O'Keefe et al (1974), but this technique also revealed the absence of the EGF receptor. On the other hand, Traish + Wotiz (1987) using identical radio ligand technique was able to measure EGF receptors in the rat prostate convincingly. This was a confirmation of speculations made by previous workers that EGF receptors might be present in the rat prostate (McKeehan et al, 1984; Smith et al, 1985).

Here again, the EGF receptors have been measured in the rat prostate, but their presence could not be demonstrated in the human prostate. Does this reflect a lower quantity of the receptors in the human prostate than there is in the rat's? Or does it mean the receptors are not present at all in the human prostate? To address these questions, the author has decided to investigate again the human prostate for the presence of the EGF receptors.

However, in another experiment, Gregory et al (1986) measured large quantities of urogastrone EGF in the human prostate fluid using radioimmunoassay method. When they compared the levels of the ligand in BPH and normal prostatic fluids a clear-cut difference between the two groups was revealed. The levels in BPH were half those found in the normal age matched controls. This report was the first to establish the presence of EGF in the human prostatic fluid, which is

closely associated with the prostate tissue. The significance of this finding has not yet been established. Probably successful localisation of the receptors in the prostate may provide a link between the ligand in the prostatic fluid and the prostate, since the biological action of EGF is thought to be mediated via its receptor (Hollenberg and Cuatrecasas, 1973).

Since the development of the monoclonal antibody specific to the EGF receptor sites (Waterfield et al, 1982), no-one has used it to detect the presence of the receptor in the human prostate. However, it has been successfully used to measure EGF receptors in a whole range of other tissues (Hendler + Ozanne, 1984); Sainsbury et al, 1985; Lau et al, 1988) to mention only a few, employing the technique of immunocytochemistry.

In the meantime, Koutsilieris et al (1987) also recently isolated two peptides of molecular weights of 13,000 and 10,000 daltons from BPH, but found that the 10,000 dalton mol. weight was only associated with adenocarcinoma tissues. These peptides were also found to be different from those previously described (Jinno et al, 1986; Maehama et al, 1986) in terms of biochemical composition. Both peptides were thought to be implicated in osteoblastic metastasis since they increased cell numbers and alkaline phosphatase activity in osteoblast-like cells with effects on both growth and differentiation.

It would seem that the prostate is full of growth factors; some are already known and characterised, others have just been described. Their real identities and role in the development of BPH and CaP are yet to be established. Yet new ones are still being added to the list as the days unfold. This truly underscores the concept that multiple peptide growth factors are necessary for tissue growth (Sporn and Roberts, 1988). These authors further suggested that the use of combinations selected from a larger number of peptide

signalling molecules, increases the amount of information that can be transmitted.

Another piece of work which related to the human prostate was undertaken very recently by Shuurmans et al (1988). They showed that androgens stimulated both growth rate and EGF receptor activity of the human prostate tumour cell (LNCaP). This suggests the presence of EGF receptors in the human prostate tumour cells. Furthermore, using radio ligand technique, they demonstrated the presence of the receptors in the tumour cells. But results obtained from such cell lines should be interpreted with caution. These cell lines have been passaged over the years. It is possible that they might have undergone phenotypic changes which could have important implications on the EGF receptor expression.

The literature suggests that EGF is present in the guinea pig and rat prostate but in the human prostate, either it is there in smaller quantities, or the ligand is not there at all. Furthermore, the presence of the EGF receptors in the rat prostate has been demonstrated by a number of biochemical techniques, but in the human prostate tissue the presence of the EGF receptors is still not clearly defined. The uncertainty about the presence of the receptors for EGF in the human prostate tissue makes it difficult to assess the status of the ligand in the gland.

However, the presence of both ligand and the receptor in the rat prostate argues for a role for the ligand in the rat prostate. It is therefore speculated that EGF may have a similar role in the human prostate in view of the large quantities of EGF found in the human prostatic fluid. But it is clear now, that even if EGF has a role in the prostate with regard to BPH development, and CaP, it may not be functioning alone. The existence of other growth factors, e.g. NGF in the guinea pig prostate

(Harper et al, 1979) and the discovery of other mitogenic factors in both the animal and human prostates, clearly shows that these other peptides might be functioning in concert with EGF.

In the human, as well as the animal models, the only way to demonstrate, if EGF has any role in BPH or CaP development, is to establish the presence of the ligand or its receptor in the tissue. It was therefore decided to combine the two techniques of immunocytochemistry involving the use of the monoclonal antibody to the receptor site now available and radioligand assay to localise and characterise the EGF receptor in the human prostate. The author is of the opinion that the use of the two techniques should provide adequate guarantee for the detection of the receptors.

It is speculated that the level of EGF receptor or EGF expression may vary from one tissue to another. In some cases, it is possible that the quantity may be so low as to fall below the detection limit of a less sensitive technique used. If this happens, that tissue may be easily classified as negative.

The reasons for undertaking this research are now summarised:-

a) It is hoped that the localisation of the EGF receptors in the human prostate tissue may provide a link between the large quantities of urogastrone EGF in the human prostatic fluid and the human prostate tissue. This, it is hoped, may also provide explanation for the high and low levels of the ligand observed in normal and BPH prostatic fluids respectively.

b) The improved sensitivity of immunocytochemistry, due to availability of monoclonal antibodies to the external and internal domains of the EGF receptor, prompted many workers to use the technique, involving the monoclonal antibodies, to measure EGF receptors in a whole range of

tissues. But the fact that no-one has used the technique involving the monoclonal antibodies on the human prostate tissue, prompted the author to use it to measure and localise the EGF receptor in the human prostate.

c) Furthermore, EGF and its receptor are convincingly established in the rat prostate. This argues for a role for the ligand in the rat prostate. It is speculated that a similar role for the ligand may operate in the human prostate. Localisation of the EGF receptor or the ligand in the human prostate tissue may help to throw light on this speculation. Furthermore, it may also establish that EGF activity is part of a whole array of mitogenic activities which are now known to be also present in the prostate.

## 1.5 OBJECTIVES

1. To localise and characterise the epidermal growth factor receptor in the human prostate by biochemical and immunocytochemical methods.

2. To compare EGF receptor levels between BPH and CaP.

3. To correlate EGF receptor levels with the histologic grades of CaP.

4. To demonstrate the presence of external and internal domains of the EGF receptor.

5. To further characterise the receptor by chemical cross-linking and phosphorylation approaches.

# CHAPTER 2

# MATERIALS AND METHODS

CHAPTER 2

## 2.1 MATERIALS

a) Radioactive:

i) $Na^{125}$ [(Specific Activity 350 - 600 mCi/ml)

ii) [$\gamma$ $^{32}P$] ATP 3000 Ci / mmol

were purchased from Amersham International plc, Berks.

b) Non-Radioactive:

i) Hormones and growth factors:

Mouse epidermal growth factor (EGF)

Venom nerve growth factor (VNGF)

were purchased from Sigma Ltd, Poole, Dorset, as electrophoretically pure and were used without further processing.

Human prolactic (hPRL)
Human follicle stimulating hormone (hFSH)
Human luteinizing hormone (hLH)
Human growth hormone (hGH)
h Insulin

were generously donated by NIADDK, Bethesda, MD USA.

ii) Enzymes and enzyme inhibitors:

Leupeptin, aprotinin, phenylmethylsulphonylfluoride, trypsin, antitrypsin (soybean), $\alpha$ chymotrypsin and deoxyribonuclease (DNASE)

were supplied by Sigma Ltd.

iii) Other chemicals:

Disuccinimidyl suberate (DSS) was purchased from Pierce and Warriner Chemical Co UK Ltd, 44 Northgate Street, Chester, CH1 4EF.

Electrophoresis reagents and equipment were obtained from Bio-Rad Laboratories Ltd, Caxton Way, Watford Business Park, Watford, Hertfordshire, WD1 8RP.

The antihuman EGF receptor monoclonal antibody (EGF-R1) was a generous gift from Drs M.D. Waterfield and P. Bennet of Imperial Cancer Research Fund Laboratories, London.

Monoclonal antibody to the cytoplasmic domain of the EGF receptor (MAB F4) was also a generous gift from Dr W. Gullick, Imperial Cancer Research Fund Laboratories, London.

Normal rabbit serum was obtained from Scottish Antibody Production Unit, Carluke, Lanarkshire.

Human urogastrone was purchased from Sigma Ltd and was used without purification.

Peroxidase conjugated antimouse immunoglobulin was from Dako Ltd, Glostrup, Denmark.

Diaminobenzidine (DAB) and Harris haematoxylin stain were obtained from BDH Chemicals Ltd, Poole, Dorset.

All other reagents were of analytical grade except bovine serum albumin ((BSA) fraction V) which was obtained from Sigma Ltd.

2.2 a) BUFFERS (GENERAL)

The following buffers were used throughout the investigation:-

i) Buffer A: Containing Tris (10 mmol/L), EDTA (1mmol/L), EGTA (1mmol/L), sucrose (0.25 mol/L) and phenylmethylsulphonyl-fluoride (PMSF) (0.05 mmol/L. pH 7.4).

ii) Buffer B: Containing Tris (10 mmol/L), sodium chloride (0.9% w/v) and BSA(0.1% w/v) pH 7.4.

iii) Buffer C: Containing Tris (100 mmol/L) and $MgCl_2$ (4 mol/L) pH 5.8.

iv) Buffer D: Containing Tris (25 mmol/L) $MgCl_2$ (10 mmol/L) charcoal (0.3% w/v) and dextran (0.03% w/v) pH 7.5.

v) Buffer E: Containing $Na_2HPO4.\ 2H_2O$, (0.05 mol/L), $NaH_2PO4.\ H_2O$. (0.05 mol/L) and NaCl (0.9% w/v) pH 7.4.

vi) Buffer F: Containing Tris-HCl (10 mmol/L) pH 7.4.

Note: The half life of PMSF in aqueous solution is about 30 minutes. For this reason a solution of PMSF in absolute alcohol was made up on the day of the experiment and added in appropriate amount to the buffer to give 0.05 mmol/L final concentration.

b) ELECTROPHORESIS: Buffers and Reagents

i) 30% Acrylamide containing acrylamide 30g, bisacrylamide 0.85, distilled water 100 mls.

ii) Resolving gel buffer containing Tris HCl (3M) made up of Tris (36.8g) 1N HCl (48 mls), water (100 ml) pH 8.8.

iii) Stacking gel buffer containing Tris-HCl (0.5M) made up of Tris (6.0g), 1N HCl (48 mls), water (40 ml), pH 6.8.

iv) Electrophoresis buffer containing Tris (6g), Glycine (28.8g), sodium dodecylsulphate (SDS) (2.0g), water to 2L, pH 8.5.

Preparation of gels

v) Stacking gel 3% (20 ml volume)

Acrylamide 2ml
Stacking gel buffer 5ml
10% SDS (not added)
Water 13 ml
TEMED 80 ml
10% Ammonium persulphate 120 µl

vi) Resolving gel 7½% (60 ml volume)

Acrylamide 15 mls
Resolving gel buffer 6 mls
10% SDS 0.68 ml
Water 38 mls
TEMED 200 µl
10% Ammonium persulphate 200 µl

vii) Boiling mixture

8% SDS
20% glycerol
5% β - mercaptoethanol
0.05 bromophenol blue
50 mmol/L Tris buffer (100 ml)
pH 6.8

viii) Staining solution

0.1% brilliant blue R 250 (W/V) in 20% methanol and 10% glacial acetic acid mixture.

ix) Destaining solution

20% methanol and 10% acetic acid mixture.

x) HEPES buffer preparation

| | | | | |
|---|---|---|---|---|
| Hepes | 20mM | 0.477g | ) | |
| BSA | 1% | 1.0g | ) | Dissolve in 100 mls |
| NaCl | 128 mM | 0.748g | ) | water and pH to |
| KCl | 5mM | 0.037g | ) | 7.4 using 1N NaOH |
| $MgSO_4.7H_2O$ | 1.3 mM | 0.032g | ) | |

Presence of $Ca^{++}$ (1.3 mM) is optional.

c) OTHER BUFFERS AND REAGENTS

i) Michaelis Veronal acetate buffer pH 9.2

| | |
|---|---|
| Sodium acetate trihydrate | 9.7g |
| Sodium barbitone | 14.7g |
| Distilled water | 500 mls |
| Formaldehyde | 10 drops |

To above add 4M HCl dropwise to bring pH to 9.2.

ii) Naphthol ASBI phosphate solution:- Dissolve 0.14g of naphthol ASBI Phosphate in 2 ml of dimethylformamide (DMF).

iii) 0.05 M Levamisole solution:- Dissolve 0.24g levamisole in 20 mls of distilled water.

iv) Alkaline phosphatase chromogen :- To 10 mls of Michaelis Veronal buffer pH 9.2 add:
5 mg fast red ITR
200 ul 0.05 M Levamisole
Mix and add 1 drop of naphthol ASBI phosphate in DMF
Mix well and filter onto slides
Incubate 10 - 20 minutes at room temperature

v) Immunoperoxidase staining:

Preparation of 2nd antibody: Rabbit antimouse peroxidase conjugated immunoglobulin (RAMPC) -

1720 ul NRS*/TBS⌀ 1/6 dilution
80 " RAMPC
200 " TBS containing 2mg IgG

* NRS = Normal rabbit serum

⌀ TBS = Tris buffered saline

2.3 METHODS

Iodination: The method selected involved the use of a water insoluble reagent 1, 3, 4, 6 - Tetrachloro 3α 6α diphenyl glycouril ("Iodogen") first described by Fraker and Speck (1978) as a reagent for iodinating proteins and cell membranes. It offers many advantages over chloramine T and lactoperoxidase methods.

a) Principle: "Iodogen" usually coated on the inside of reaction tubes mediates rapid iodination in the solid phase with aqueous solutions of $I^-$ and proteins.

b) Preparation of coated tubes: "Iodogen" reagent dissolved in methylene chloride or chloroform at a concentration of 150 µg/ml was pipetted into plastic Eppendorf tubes in 200 µl aliquots. Each tube was placed under a gentle stream of nitrogen gas with hand warming until all the methylene chloride was evaporated, leaving a coating of "iodogen" reagent on the inside of the tubes. Coated tubes were stored at -20°C until required.

c) Iodination procedure: Into the iodogen coated tube was pipetted 10 ug EGF contained in 100 µl of buffer B. 500 µCi/5µl of Na $^{125}I$ was used. The mixture of Na$^{125}I$ and EGF was whirl-mixed, incubated for 20 minutes and purified by column chromatography as described below.

d) Purification (by Column Chromatography): A chromatography column (1 x 46 cm) was packed with Sephadex G-50. 5g of Sephadex G-50 was soaked in 50 ml buffer E and was allowed to equilibrate overnight prior to the packing.

The mixture of Na $^{125}I$ and EGF was transferred to the column which was mounted on a retort stand. The reaction vessel was washed twice with buffer E and the washings added to the column.

e) Collection of fractions: Fractions of 1 ml/2 minutes were collected from the column into glass tubes, using a Pharmacia fraction collector programmed to collect 1 ml fractions (Fraction Collector Frac 300 Pharmacia Fine Chemicals).

f) Measurement of fractions: Each fraction was counted on a gamma counter (Crystal II Multidetector Gamma System Model 5412 efficiency 84%). When the unit counts obtained in each tube were plotted against the tube numbers, it was revealed that three distinct peaks were eluted, namely A, B, C, with A being the first to be eluted. Furthermore, binding studies with the three peaks demonstrated that the highest binding was achieved using peak B. Peaks A and C produced little or no binding at all. Peak B was therefore always kept for binding studies.

The percentage bound was calculated after precipitation with trichloroacetic acid. The final specific activity varied between 30 and 70 uCi/ug.

2.4 PROTEIN DETERMINATION: Protein determination was by the method of Bradford (1976).

a) Reagents: Coomassie Brilliant Blue G (100 mg) was dissolved in 50 ml of 95% (v/v) ethanolic solution. To this 100 ml of phosphoric acid 85% (v/v) was added and mixed. The solution was made up to 1L with distilled water and finally filtered through Whatman paper to remove insoluble material.

b) Bovine serum albumin (BSA) standard solution: A solution of BSA in distilled water (approximately 1 mg/ml) was made and its absorbance read at 280 nM, using a Pye Unicam (Model 550 UV/VIS) spectrometer which was zeroed with distilled water in a quartz cuvette. The concentration of the BSA solution was adjusted to give absorbance

reading of 0.650 units, corresponding to a protein concentration of 1 mg/ml. Aliquots of this solution were stored at -20°C until required for use.

c) BSA calibration curve: 20 ul amounts of BSA standard solutions (0-80 µg) were dispensed into standard tubes. 5 mls of Bradford reagent were added to each tube and the contents vortexed. The spectrophotometer was zeroed (with 0µg BSA) and the contents of the tubes read for absorbance at 595 nm. Absorbance values obtained for each tube were plotted against protein concentration.

280 nm wave-length is normally used to measure amino acids with phenolic group in a standard solution, whilst 595 nm is used to measure peptide bonds in the test solution.

## 2.5 PRELIMINARY EXPERIMENTS:

a) Iodination peaks: Figure 7 shows a typical pattern obtained after plotting unit counts/tube against fraction number as detailed in 2.3 (a) - (f). Three distinct peaks were revealed (A, B and C). After using an aliquot from each peak for binding studies, only peak B showed high specific EGF binding. Peak B was therefore kept at 4°C for subsequent use.

b) Protein standard calibration curve: Figure 8 shows a typical pattern of protein standard calibration curve. When concentration against absorbance was read at 595 nm, it was found that the curve was linear over a protein range of 0 - 80 µg BSA. In excess of 80 µg BSA, a plateau forms. Details of the technique are described under 2.4 (a) - c)).

Figure 7.

Iodination procedures

10 µg EGF was iodinated with 500 uCi of Na $^{125}I$ in iodo-gen coated tubes. The iodinated EGF was purified by column chromatography.

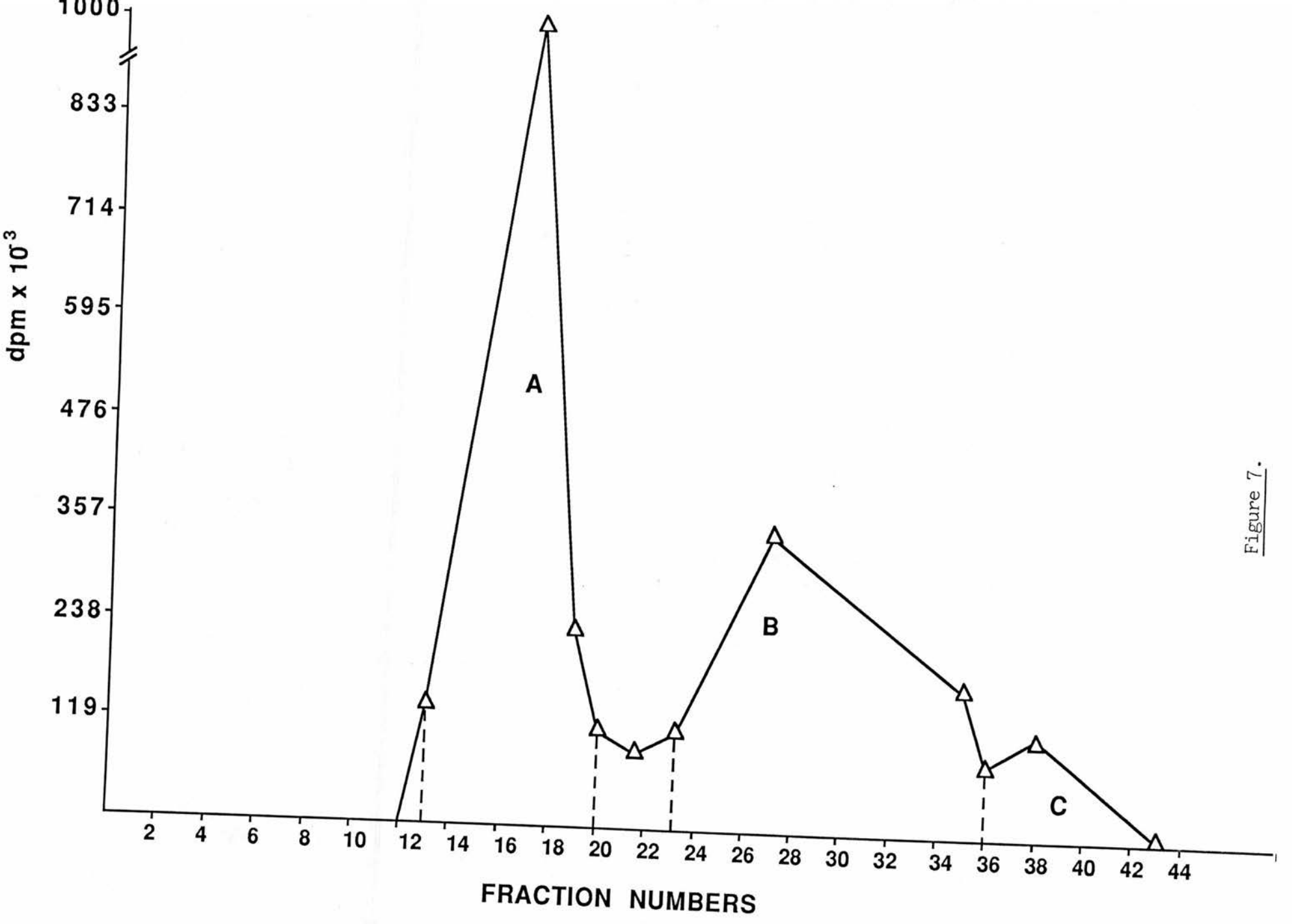

1000
833
714
595
476
357
238
119
dpm x $10^{-3}$
A
B
C
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
FRACTION NUMBERS

Figure 7.

Figure 8.

Protein calibration curve

Increasing amounts (10 - 80 μg) of a BSA solution (1.0 mg/ml) were mixed with Bradford reagent and read for absorbance at 595 nM.

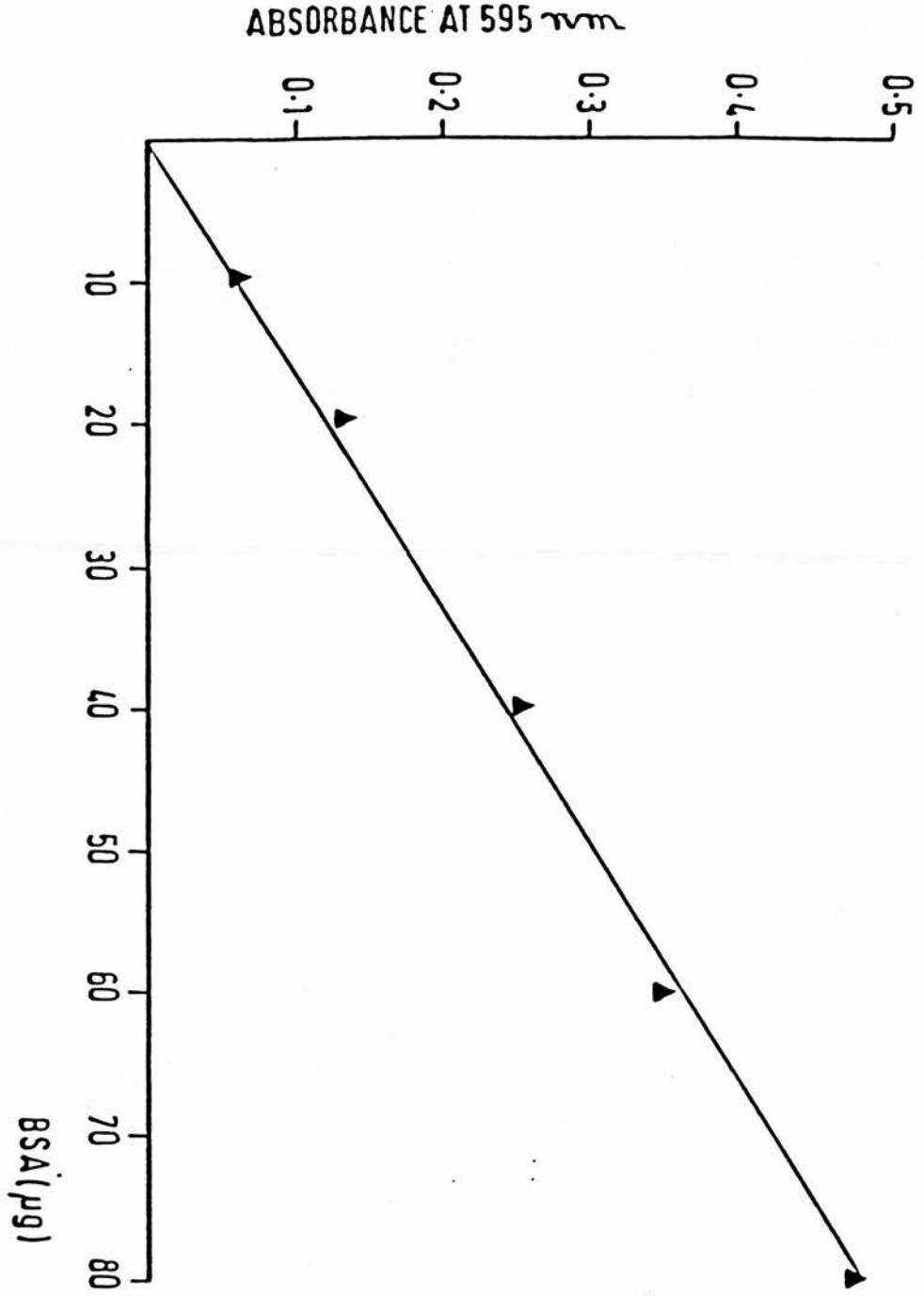



Figure 8.

## 2.6 SELECTION AND PREPARATION OF TISSUES AND STORAGE

a) Prostate (General procedures); Human prostate clinically and histologically diagnosed as hyperplastic or malignant was removed by transurethral resection (TUR) or open prostatectomy and transported to the laboratory in ice-cold saline (0.9% w/v). The tissue was washed three times with Tris-HCl buffer pH 7.4 (buffer F) and blotted dry with tissue paper. Prostatic tissue removed by open prostatectomy was cut into 2 gm blocks and then divided into 2 - 3 blocks per glass universal bottle. All tissues were snap frozen in liquid nitrogen and stored at -70°C until required. In the case of TUR all burnt areas of prostate chips were carefully removed and only the clean juicy tissues not affected by resection loop were stored.

b) Prostate: Selection of benign prostatic hyperplasia (BPH) and cancer of the prostate (CaP) tissues:

Separate prostate specimens were taken from 18 patients with BPH, and 19 patients with CaP. None of the patients from whom the tumour samples were obtained received chemotherapy or radiation prior to the removal of the tissues. Histopathologically the cancerous specimens consisted of 4 well differentiated, 5 moderately differentiated and 10 poorly differentiated tumours.

Grading was carried out by a single pathologist according to the Gleason system (Gleason, 1966).

The specimens were immediately frozen in liquid nitrogen and kept at -70°C pending analysis.

c) Placental tissue: Term human placenta from spontaneous normal deliveries were brought to the laboratory on ice. The tissues were scraped off from the underlying blood vessels and repeatedly washed with chilled physiological saline to remove blood. It was either

used fresh, or snap frozen in liquid nitrogen and kept at -70°C.

d) Plasma: Preparation: Blood drawn from patients diagnosed with benign prostatic hyperplasia was collected in containers with heparin anti-coagulant and delivered to the laboratory on ice. The blood was transferred to low speed centrifuge tubes and cells removed at 800 x g for 15 minutes. The clear supernatant (plasma) was aspirated and divided into 1 ml aliquots for storage at -20°C.

2.7 PREPARATION AND HOMOGENISATION OF TISSUE

a) The procedure is the modified version of the method described by Hock+Hollenberg(1980) and Mukku+Stancel (1985) and Sainsbury et al (1985).

Briefly, about 1 - 2g tissue was washed in buffer A, blotted dry and cut into small pieces with scissors. Unless specified, all procedures were carried out at 4°C. Preliminary dispersion of the tissue was carried out on a microdismembrator (B. Braun, A.G. Melsungen FRG) for 20 seconds in a prechilled teflon container. The tissue was further homogenised in 3 vols of buffer A using Ystral homogeniser (Scottish Scientific Instruments Centre Ltd, Edinburgh) for two periods 20s and 15s respectively at position 6 with 1min cooling intervals. The homogenate was subsequently filtered through a metal strainer and the filtrate dispersed into 10 ml capacity polycarbonate centrifuge tubes (Sorvall, Herts, U.K.) which were loaded in an angled T865.1 rotor (Dupont Instruments, Herts, U.K.). Ultracentrifugation was carried out at 105,000 xg for 40 minutes. The resultant pellet (total particulate fraction) was resuspended in buffer A and dispersed further in a glass Dounce homogeniser using 50 strokes with the loose fitting pestle followed by a second ultracentrifugation at 105,000 x g for 20 minutes. The resultant pellet was suspended in buffer B followed

by 10 strokes with the tight fitting pestle. The protein concentration of the final particulate fraction was measured by the method of Bradford (1976). The protein concentration in the particulate fraction was then adjusted to 1 mg and stored at -70°C.

b) Method of membrane preparation for molecular characterisation of the EGF receptor

i) For crosslinking experiments: The method was based on the technique described under 2.7(a) with slight modifications.

Briefly, homogenate preparation was centrifuged at 800 x g for 10 minutes. The supernatant was saved. The pellet was resuspended in buffer B and the centifugation process repeated. The two 800 x g supernatants were combined and centrifuged at 105,000 x g for 60 minutes. The pellet was suspended in buffer B and protein concentration adjusted as described under 2.7(a). 1ml aliquots containing 1 mg protein were stored at -70°C for subsequent use.

ii) For phosphorylation experiments: Homogenates designed for phosphorylation experiments were stored in 1 ml aliquots containing 6 - 8 mg protein at -70°C.

c) Subcellular fractionation: Subcellular fractions were prepared according to the method of Leake et al (1983) with slight modification.

Briefly, prostate homogenate was prepared as described under 2.7(a). The homogenate was centrifuged at 800g for 20 minutes to obtain a crude heavy pellet. The supernatant was also centrifuged at 15,000g for 20 minutes to obtain the mitochondrial pellets. The supernatant resulting from this was centrifuged at 105,000g to obtain a pellet for the microsomal fraction and the 105,000g supernatant fraction (cytosol). The pellets were reconstituted in 1 ml of buffer B and were either used immediately or stored at -70°C until required.

## 2.8 ASSAY OF TISSUE PROTEIN

This was based on methods of Bradford (1976) and Mukku+Stancel (1985) Equal volumes of particulate fraction and 2N NaOH were boiled for 15 minutes. The mixture was diluted with an equal volume of distilled water and allowed to cool. 20 µl of mixture was added to 5 mls of Bradford reagent and mixed. 20 µl of standard solutions containing 0 - 80 ug protein were added to 5 mls of Bradford solution and mixed. The spectrophomoter was zeroed using 20 µl distilled water and 5 mls Bradford reagent. A calibration curve was constructed using the absorbance at 595 nm against standard protein concentrations. The value of the homogenate protein suspension was read off the standard protein calibration curve described under 2.4(c).

## 2.9 $^{125}$I-EGF BINDING WITH BPH

Binding of EGF was determined by a modification of the methods of Carpenter and Cohen (1975; 1979), Sainsbury et al (1985) and Mukku and Stancel (1985).

Briefly, 100 µl homogenate samples were incubated with 100 ul $^{125}$I-EGF solution at a one point concentration in the $10^{-9}$M range in the presence and absence of 50-fold excess unlabelled EGF. The final volume of the incubation mixture was 400 µl made up of 200 µl buffer B without unlabelled EGF and 100 µl $^{125}$I-EGF and 100 ul sample. Incubation took place at 4°C, 25°C and 37°C respectively. The reaction was terminated after 40 minutes by adding 1 ml cold (4°C) buffer B. The samples were harvested by filtration under reduced pressure on glass microfibre filters (Whatman GF/A, Whatman Ltd, Maidstone, Kent) and rapidly washed with aliquots of buffer B. The radioactivity retained by the filter was measured on a gamma counter (Crystal II Multidetector Gamma System, Packard Co) with an

efficiency of 84%. The specific binding was calculated by subtraction of non-specific from the total binding.

## 2.10 SEPARATION OF SPECIFIC EGF BINDING FROM NON-SPECIFIC

Experiments described in this section were aimed at selecting suitable technique for separating specific binding from non-specific.

The techniques are now described:-

a) Filtration and washing.

b) Filtration.

c) PEG precipitation: Aspiration and pouring.

d) PEG precipitation: Centrifugation.

a) Effect of filtration and washing on: Binding of $^{125}$I-EGF to BPH particulate fraction: This was based on the method described under 2.9.

Briefly, four 100 µl aliquots of particulate fraction were incubated with $^{125}$I-EGF in the $10^{-9}$M concentration range in the presence and absence of 50-fold excess unlabelled EGF at 37°C for 40 minutes. At the end of the incubation, the reaction was terminated by the addition of 1 ml cold (4°C) buffer B to each of the four incubation mixtures. The samples were harvested by filtration under reduced pressure on glass microfibre filters (Whatman GF/A, Whatman Ltd, Maidstone, Kent). Thereafter radioactivity retained on the filter paper was washed once, twice, thrice or four times using 1 ml buffer B in each case. The final radioactivity retained on the filter paper was measured on a gamma counter.

The specific binding was calculated by subtraction of non-specific binding from total.

Relationship between specific binding and the effect of washes on pellet retained on filter was established.

b) Filtration technique and polyethylene glycol (PEG) precipitation technique compared

The filtration method is described under 2.9.

The PEG technique is a modification of the method of Hwang et al (1986). The receptor binding assay was essentially the same as described under 2.9.

Briefly, after an incubation of $^{125}$I-EGF with prostate particulate fraction, the $^{125}$I-EGF receptor complex was precipitated with 1 ml 20% PEG in buffer B at 4$^{o}$C and allowed to stand for 10 minutes. The mixture was centrifuged at 3000 rpm for 20 minutes and the pellet washed once with 10% PEG and 0.05% BSA and centrifuged again as before. The bound radioactivity retained in the pellet was measured by a gamma counter.

Relationship between the level of specific binding obtained by the filtration technique and the PEG method was established.

c) PEG precipitation methods of removing free $^{125}$I-EGF investigated. Pouring compared with aspiration

The receptor binding assay was the same as described under 2.9. At the end of the incubation period, the $^{125}$I-EGF participate fraction bound complex was precipitated by 20% PEG and centrifuged.

In one set of experiments the supernatant was poured off touching the tip of the reaction tube on a tissue.

In another set of experiments the supernatant was aspirated off using a venturi pump which provided a negative pressure. The process was repeated after washing the pellet once with 10% PEG. Bound radioactivity retained by each method was measured on a gamma counter.

Comparison between the level of specific binding achieved by the pouring technique and the aspiration method was made.

d) PEG precipitation: duration of centrifugation

The receptor binding assay was identical as described under 2.9. The $^{125}$I-EGF bound complex was precipitated using 20% (w/v) PEG in buffer B. The mixture was centrifuged at 3000 rpm for 5, 10, 15, 20, 30 and 45 minutes in 6 separate experiments. After washing the pellets with 10% (w/v) PEG and 0.05% BSA the mixture was again centrifuged at 3000 rpm, for the same periods of time, and supernatant again discarded. Specific radioactivity retained after each period of centrifugation was measured on the gamma counter.

Relationship between specific binding and duration of centrifugation of $^{125}$I-EGF bound complex was established.

## 2.11 VALIDATION OF THE ASSAY FOR EGF RECEPTOR

a) The human placenta was used because it is a tissue known to contain EGF receptors (Hock+Hollenberg1980; Ramani et al (1986).

i) Measurement of EGF urogastrone binding: The binding method was based on modifications of the methods of Hock+Hollenberg(1980); Sainsbury et al (1985); Ramani et al (1986); Hwang et al (1986)

Briefly, placenta 105,000g particulate fractions were incubated with $^{125}$I-EGF solution at one point concentration in the $10^{-9}$M range in the presence and absence of excess unlabelled EGF. The final volume of the incubation mixture was 400 µl, made up of 200 µl buffer B, with or without unlabelled EGF, 100 µl $^{125}$I-EGF and 100 µl sample. Incubation took place at 37°C for 40 minutes and the reaction was terminated by the addition of 20% polyethylene glycol (PEG) in buffer B to precipitate the $^{125}$I-EGF receptor complex, at 4°C for 10 minutes. The mixture was centrifuged at 3000 rpm for 20 minutes and the pellet washed once with 10% PEG and 0.05% BSA and centrifuged again as before.

The supernatant was aspirated each time. The bound radioactivity retained in the pellet was measured on a gamma counter. Specific binding was calculated by the difference between total binding and non-specific binding.

ii) Saturation studies: Placental membrane aliquots were incubated with increasing concentrations of $^{125}$I-EGF (0.5 - 12nM) in the presence and absence of excess unlabelled EGF. After 40 minutes incubation at 37°C and PEG precipitation to separate bound complex from free, the specific binding was calculated as above, i.e. by the difference between total and non-specific binding.

iii) Scatchard plot analysis: Data obtained from the saturation studies outlined above was used in calculating the dissociation constant (Kd) and estimating the number of binding sites of the receptor by the method of Scatchard (1949).

iv) The outcome of the validation experiments: TABLE 1 indicates that the human placenta contains a high level of EGF receptors, as demonstrated by the level of specific binding/mg of protein.

v) Saturation studies: Human placenta: Figure 9 demonstrates the relationship between specific binding and concentration of $^{125}$I-EGF. It is observed that saturation of the binding occurs from 6.0nm m/l of $^{125}$I-EGF concentration which is indicated by plateau formation.

vi) Scatchard analysis on placenta binding: Figure 10 shows the Scatchard plot obtained from the saturation analysis, a single plot indicating one class of binding site of high affinity of $0.38 \times 10^{-9}$ M.

TABLE 1

EGF BINDING TO HUMAN PLACENTA

The method of the assay was as described under section 2.11(a).

6.0 nm $^{125}$I-EGF was incubated with placenta particulate fraction (1 mg protein/ml) in the presence of 50-fold excess unlabelled EGF. Non-specific binding was determined by running a parallel assay in absence of unlabelled EGF. Specific binding was determined after PEG precipitation and subtraction of non-specific from total binding.

Values were means of four different experiments, each analysed in duplicate.

TABLE 1.

| | | | | |
|---|---|---|---|---|
| **TOTAL BINDING** | **=** | **6467 dpm** | **±** | **100 dpm** |
| **NON SPECIFIC BINDING** | **=** | **250 dpm** | **±** | **50 dpm** |
| **SPECIFIC BINDING** | **=** | **6217 dpm** | **±** | **100 dpm** |

Figure 9

Saturation Studies: Human Placenta

Briefly, $^{125}$I-EGF in a range of concentrations from 0.5 - 12nmol/L were incubated with placenta particulate fraction in the presence and absence of unlabelled EGF at 37$^{o}$C for 40 minutes.

Separation of $^{125}$I-EGF bound complex from free and subsequent calculation of specific binding was essentially as described under 2.11(a)(i).

Values were means of 4 different experiments, each analysed in duplicate.

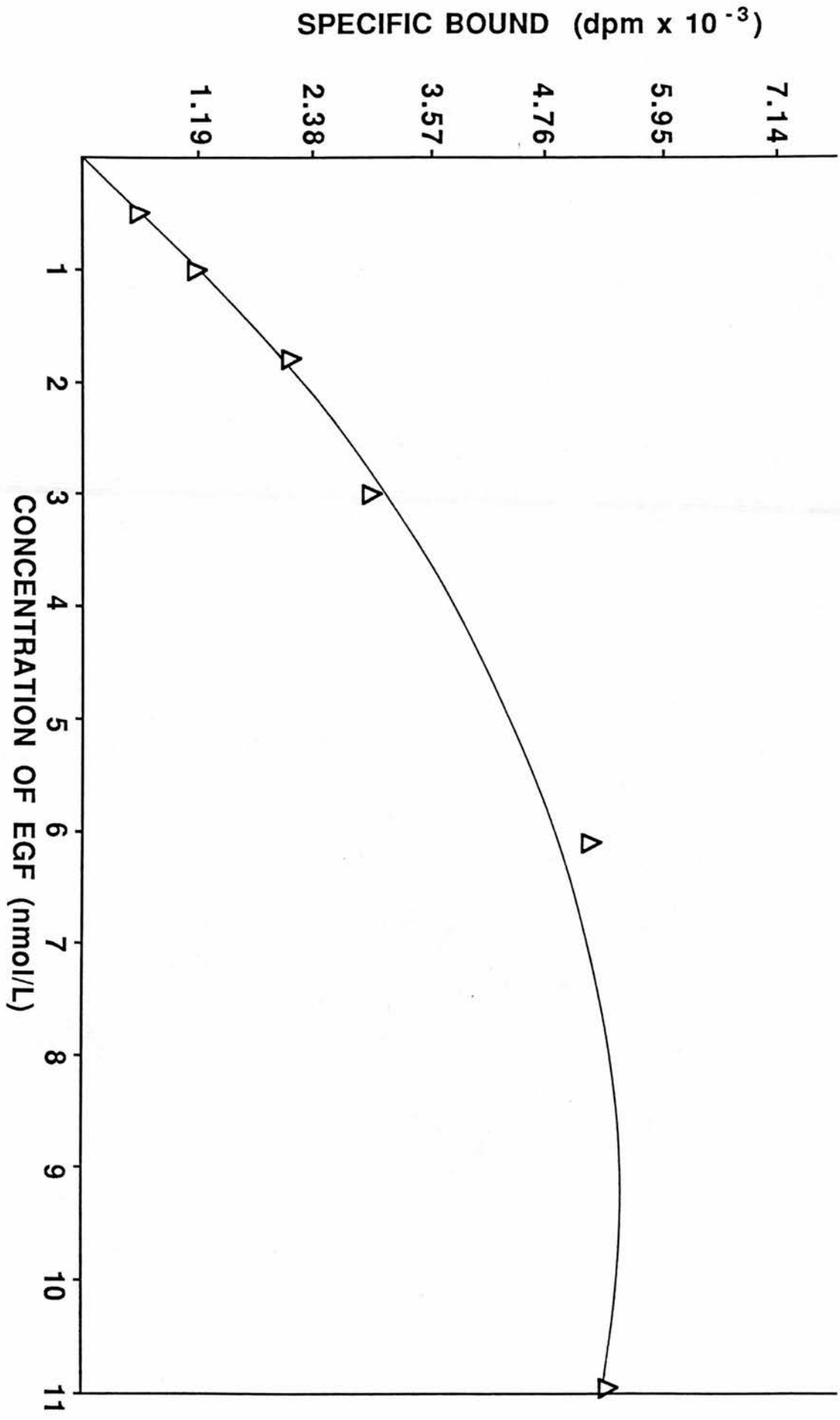

SPECIFIC BOUND (dpm x 10 -3)
1.19
2.38
3.57
4.76
5.95
7.14
CONCENTRATION OF EGF (nmol/L)
1
2
3
4
5
6
7
8
9
10
11

Figure 9.

Figure 10

Scatchard analysis on placenta binding

Data obtained from the saturation studies as described under section 2.11(a)(ii) was used in calculating the dissociation constant (Kd), using the method of Scatchard (1949).

Values were means of 4 different experiments, each analysed in duplicate.

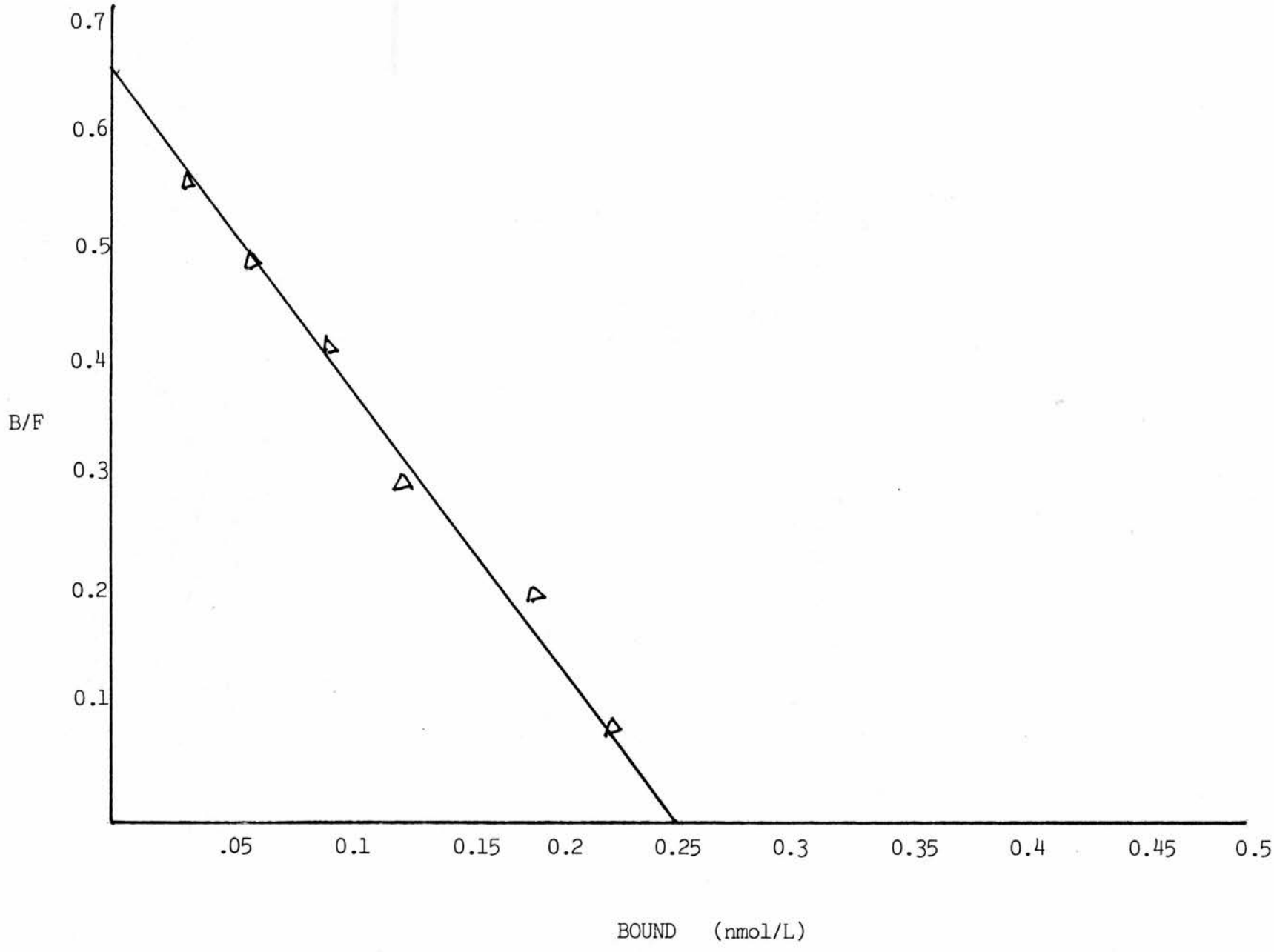

0.7
0.6
0.5
0.4
0.3
0.2
0.1
B/F
.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4
0.45
0.5
BOUND (nmol/L)

Figure 10.

These experiments demonstrated that the $^{125}$I-EGF binding technique was working well.

b) Binding with human plasma

100 µl of $^{125}$I-EGF in the $10^{-9}$M concentration range was incubated with 100 µl of plasma diluted with buffer B to give 1 - 5 mg/ml protein in the presence of excess unlabelled EGF. Non-specific binding was determined by running a parallel assay in the presence of unlabelled EGF. Specific binding was calculated by subtracting non-specific from total binding as described under section 2.11.

TABLE 2 indicates that no specific binding was observed when $^{125}$I-EGF reacted with human plasma.

## 2.12 CHARACTERISATION OF THE EGF RECEPTOR

After the selection of a suitable method for separating specific binding from non-specific, it was next decided to characterise the EGF receptor by the following biochemical techniques described in this section:-

a) Subcellular distribution of the EGF receptors in BPH

Subcellular fractions were prepared according to a modified version of the method of Leake et al (1983) and this is detailed in section 2.7(c).

Briefly, prostate homogenate was centrifuged at 800g for 20 minutes to obtain crude heavy pellet. This was followed by centrifugation of the supernatant at 15,000g for 20 minutes to obtain the mitochondrial pellet. Finally, the supernatant resulting from this was spun at 105,000g for 40 minutes to obtain the microsomal

TABLE 2

EGF BINDING TO PLASMA

TABLE 2 indicates that no specific binding was observed when $^{125}$I-EGF reacted with human plasma.

Values were means of 4 different specimens, each analysed in duplicate. Bound complex was separated from free by the PEG method.

TABLE 2.

| | | | | |
|---|---|---|---|---|
| **TOTAL BINDING** | **=** | **1456** | **±** | **40** |
| **NON SPECIFIC BINDING** | **=** | **1560** | **±** | **45** |
| **SPECIFIC BINDING** | **=** | **-** | **-** | **-** |

pellet and cytosol. Each pellet was reconstituted in 1 ml buffer B for EGF receptor binding. 100 µl aliquots of the resuspended pellets, (i.e. 800g, 15,000g, 105,000g) and the cytosol were incubated with 100 µl $^{125}$I-EGF in the $10^{-9}$M concentration range in the presence and absence of 50-fold excess unlabelled EGF. $^{125}$I-EGF bound complexes were separated by PEG precipitation and centrifugation as described under Section 2.12. Specific binding shown by each fraction was calculated by the difference between non-specific and total binding.

b) Effect of protein concentration on receptor estimations:

Protein concentration in the BPH particulate fraction was measured as described under section 2.8.

Briefly, particulate fraction suspension was adjusted with buffer B to give protein concentrations in the range 0.1 - 8 mg/ml. 100 µl of suspension containing each protein concentration was incubated with $^{125}$I-EGF in the $10^{-9}$M range concentration in the presence of a 50-fold excess of unlabelled EGF for 40 minutes at 37°C.

Non-specific binding was assessed by running parallel incubations in the absence of unlabelled EGF. Specific binding was calculated by subtracting non-specific binding from total.

Relationship between radioligand uptake and particulate fraction protein concentration was established.

c) Effect of time and temperature on binding of $^{125}$I-EGF to BPH particulate fraction:

EGF receptor binding assay was essentially as described under

2.11 and in this report.

Briefly, $^{125}$I-EGF in the $10^{-9}$M concentration range was incubated with total particulate fraction (105,000g pellet) prepared from human BPH. 100µl aliquots of the pellet suspensions (1 mg/ml protein) were incubated in the presence and absence of 50-fold excess unlabelled EGF at 4°C, 25°C and 37°C for a period ranging from 0 - 360 minutes. Specific binding obtained for each temperature and time were calculated after separation of $^{125}$I-EGF bound complex from free and counting on gamma counter (2.11).

Relationship between the level of binding achieved in relation to time and temperature was established.

d) Dissociation of particulate fraction bound $^{125}$I-EGF in the presence and absence of excess unlabelled EGF in medium of incubation mixture:

100 µl aliquots of particulate fraction (1 mg/ml protein) were incubated with $^{125}$I-EGF as described under 2.11. The unbound $^{125}$I-EGF was removed by centrifugation at 3000 rpm for 20 minutes. The pellets containing bound $^{125}$I-EGF were resuspended in 100 µl buffer B. Samples were diluted 4-fold with buffer B containing no unlabelled EGF or 50-fold excess unlabelled EGF and then incubated for indicated lengths of time at 37°C up to 180 minutes. The controls were precipitated with PEG immediately after dilution and bound and free separated. The specific binding in this tube was considered 100%.

This method was based on the method of Ramani et al (1986).

e) Saturation analysis: The method was based on modification of the technique described under 2.11(a)(ii).

Briefly, the binding assay was performed using $^{125}$I-EGF over a range of 0.5 - 24 nmol/L and BPH particulate fraction (1 mg/ml protein)

in the presence and absence of 50-fold excess unlabelled EGF at each concentration of $^{125}$I-EGF.

After PEG precipitation and counting, specific binding at each concentration of $^{125}$I-EGF was obtained by subtracting the non-specific binding from total.

Relationship between specific binding and concentrations of $^{125}$I-EGF was established.

f) Scatchard analysis: Data obtained from the saturation studies detailed under 2.12(e) was used in calculating the dissociation constant (Kd) and estimating the number of binding sites of the receptor protein by the method of Scatchard (1949).

g) Competition studies: $^{125}$I-EGF 8.0 nmol/L was incubated with 100 µl aliquots of BPH particulate fraction (1 mg/ml protein) in the presence and absence of 250 - 3000 ng/ml of the following unlabelled competitors: human growth hormone (h GH) h FSH, human prolactin, human insulin, human LH, mouse EGF (mEGF), venom nerve growth factor at 37°C for 90 minutes.

At the end of the incubation, bound complex was separated from free $^{125}$I-EGF and specific binding was calculated as described under 2.11 after counting on gamma counter.

The degree of inhibition of specific binding by each competitor was established.

h) The effect of pH of incubation media on $^{125}$I-EGF binding to BPH particulate fraction:

The method was a modification of the method of Ramani et al (1986).

Briefly, 100 µl samples of particulate fraction (1 mg/ml protein) were incubated for 90 minutes at 37°C with 8 nM $^{125}$I-EGF in the presence and absence of 50-fold excess unlabelled EGF in media of various pH values.

10 mM Tris-HCl was adjusted to give pH values of 2 - 11. The particulate fractions incubated at pH 7.4 served as controls. At the end of the incubation period, bound complex was separated from free by PEG precipitation and aspiration of supernatant as described under Chapter 2.11(a)(i) described by Hwang et al (1986). Specific binding was subsequently calculated by the difference between total and non-specific binding.

Relationship between specific binding and pH of reaction medium was established.

i) Thermal sensitivity of EGF binding sites in BPH particulate fractions:

Particulate fractions containing 1 mg/ml protein were preincubated for 10 minutes at 45°C, 65°C, 75°C and 95°C. After cooling the tubes, 100 µl aliquots containing 100 µg protein were tested for binding at 37°C for 90 minutes with 8.0 nmol/L $^{125}$I-EGF in the presence and absence of 50-fold excess unlabelled EGF. Particulate fraction preincubated at 37°C for 10 minutes served as control. Specific binding was calculated according to methods described under section 2.11.

In another experiment particulate fractions were incubated with 8.0 nM $^{125}$I-EGF at 65°C, 75°C and 95°C for 60 minutes. Specific binding profiles were noted.

j) Effect of pretreatment of particulate fraction with different enzymes and enzyme inhibitors on subsequent $^{125}$I-EGF

The method was based on modification of the method of Ramani et al (1986).

Briefly, 100 µl BPH particulate fraction (1 mg/ml protein)

were pretreated with enzymes or enzyme inhibitors for 1 hour at 37°C. They were centrifuged (3000 rpm 20 minutes), washed once and resuspended in 100 µl of buffer B. The particulate fractions were tested for binding at 37°C for 90 minutes with 8.0 nmol/L $^{125}$I-EGF (final concentration). The particulate fraction (1 mg/ml protein) used for controls were pretreated without enzymes but subjected to the same steps as the treatment tubes. After incubation, separation of bound from free $^{125}$I-EGF and subsequent calculation of specific binding after counting, was as described under section 2.11.

The following amounts of enzymes and enzyme inhibitors were used:- 1 mg trypsin, 1mg DNASE, 2 mg soybean (trypsin inhibitor), 1 mg phenylmethylsulphonylfluoride, 0.1 mg aprotinin, 0.05 leupeptin, 1 mg ∝ chymotrypsin, all per mg of protein.

Relationship between specific binding and the effect of enzyme or enzyme inhibitor was established.

k) Pretreatment with $MgCl_2$ and dextran coated charcoal (DCC):

This method was based on the technique of Leake et al (1983).

Briefly, BPH particulate fraction was mixed with 2 ml buffer C containing 4 M $MgCl_2$ (2.2c) at room temperature for 10 minutes. The particulate fraction suspensions were subsequently centrifuged at 3000 rpm and the resultant pellets reconstituted in buffer B and the receptor binding assay performed as described below:-

For the experiments designed to determine the effect of dextran coated charcoal on EGF binding, particulate fraction suspensions were exposed to 0.2 ml buffer D containing DCC (2.2d) for 10 minutes at room temperature. The suspensions were subsequently layered on buffer B

and centrifuged at 800 rpm for 10 minutes, to remove the DCC.

Receptor binding assay was carried out by incubating 100 µl of supernatant or reconstituted pellet with 8.0 nmol/L $^{125}$I-EGF in the presence and absence of 50-fold excess unlabelled EGF at 37°C for 90 minutes. Separation of non-specific from total binding was achieved by PEG precipitation technique (2.11). Specific binding was subsequently calculated as described under section 2.11.

Relationship between specific binding and effect of 4 M $MgCl_2$ and DCC was established.

1) Effect of storage at -70°C on prostate tissue and particulate fraction:

The method was based on modification of the method of Boyd (1985).

Briefly, a fresh prostate tissue, removed by retropubic method, was prepared as described under section 2.7(a). A portion of the tissue was homogenised immediately, and protein content was adjusted to 1 mg/ml.

The remaining tissue was divided into two halves. One half was homogenised and protein level adjusted to 1 mg/ml with buffer B. 0.5 ml aliquots of the homogenised preparation were dispensed into Eppendorf tubes. The other half of the tissue was also cut into very small portions with scissors. The well mixed mixture was divided up into 1g portions. Each portion was placed in a Universal container and frozen in liquid nitrogen. The Eppendorf tubes containing the homogenate and the Universal containers with the mixed prostate portions were stored at -70°C. EGF receptor assays were performed on the homogenate and the minced tissues after 24 hours, 1 week, 2 weeks, 4 weeks, 6 weeks, 2 months and 4 months by the method

described under section 2.11(a)(i). Specific binding obtained after each period of storage was calculated.

Relationship between specific binding and effect of storage was established.

m) Comparison of EGF and urogastrone binding to BPH particulate fraction:

100 µl 8.0 nmol/L $^{125}$I-EGF was incubated with 100 µl particulate suspension (1 mg/ml protein) in the presence and absence of 50-fold excess unlabelled EGF or urogastrone (purchased from Sigma Ltd, Poole, Dorset).

In another experiment, 8.0 nmol/L $^{125}$I-urogastrone was used in place of $^{125}$I-EGF in the presence and absence of 50-fold excess unlabelled EGF or urogastrone. Incubation took place at 37$^{o}$C for 90 minutes. Thereafter, separation of bound complex from free ligand was achieved by PEG precipitation. Specific binding was subsequently calculated as described under section 2.11(a).

Specific binding achieved by each ligand was compared.

## 2.13 MOLECULAR CHARACTERISATION OF THE EGF RECEPTOR (EGF-R)

This was carried out by the following methods: a) Chemical crosslinking of EGF to its receptor and establishment of the molecular weight of the receptor; b) Stimulation of EGF receptor phosphorylation by EGF.

a) Affinity labelling and crosslinking experiments:

The method for the experiments was based on modification of the techniques of Mukku+Stancel (1985) and Fanger et al (1986). The procedure involved a number of steps which are described below:-

i) Affinity labelling: Briefly, 1 ml aliquots of BPH membrane suspensions (1 mg/ml protein) prepared as described under 2.7(b)(i) in Eppendorf tubes were centrifuged at 14,000g for 10 minutes on Mini Centaur Centrifuge (MSE) at $4^{o}C$.

The supernatants were discarded and pellets reconstituted in 200 µl HEPES buffer. 100 µl aliquots were transferred into second Eppendorf tubes to be incubated in the presence of excess unlabelled EGF. 100 µl aliquots of 8 nMol/L $^{125}$I-EGF were placed into both tubes. One tube received 200 µl HEPES buffer containing 50-fold excess unlabelled EGF whilst the other tube received 200 µl buffer. After mixing and incubation for 90 minutes at $37^{o}C$, unbound complex was separated by centrifugation at 14,000g for 10 minutes after addition of 1 ml cold ($4^{o}C$) HEPES buffer.

ii) Crosslinking with disuccinimidylsuberate (DSS): The pellets were reconstituted in 50 µl of 1 mM DSS dissolved in dimethylsulfoxide (DMSO) so that the final dilution of DMSO was about 5%. Crosslinking continued for 15 minutes at room temperature.

iii) Solubilisation: Thereafter 25 ul of boiling mixture (8% SDS, 20% glycerol, 5% β-Mercuptoethanol, 0.05% Bromophenol Blue R in 50mM Tris buffer pH, 6.8) was added to solubilise the receptor from the membrane. The mixture was heated at 100°C for 3 minutes and centrifuged for 5 minutes at 3000 rpm to remove undissolved complex.

iv) Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS - PAGE)

The mixture was analysed and characterised by SDS-PAGE. The procedure was based on the discontinuous Tris-glycine buffer system described by Laemmli (1970) and was performed in a vertical slab gel electrophoresis apparatus from Bio-Rad. Current was supplied to the apparatus by a Vokam power supply unit.

7.5% resolving gel with 3% stacking gel was used. A mixture of molecular weight markers (200,000, 116,000, 92,000, 65,000, 45,000 and 29,000) purchased from Sigma were run in parallel lanes. Thereafter, electrophoresis took place at 35 mA/gel for $2\frac{1}{2}$ hours at 22°C.

v) Staining and destaining: Gels were stained with Coomassie brilliant blue R250 (0.1% in 25% methanol and 10% acetic acid) for 20 minutes. The gels were destained with a mixture of 25% methanol in 10% acetic acid.

vi) Drying of gel: Gel was dried at 80°C for 2 hours, using a gel dryer obtained from Bio-Rad.

vii) Autoradiography: Autoradiography was performed at -70°C for 2 - 3 weeks using hyperfilm TM obtained from Amersham placed in a cassette with intensifying screens.

viii) Film development: Film was developed in Kodak Developer LX-24 (1/5.6 dilution) and fixed in Kodak X-ray liquid fixer FX40 (1/5 dilution).

b) EGF receptor phosphorylation: The technique was based on the methods of Mukku+ Stancel (1985) and Yarden et al (1987). The method involved a number of steps and these are described below:-

i) Preparation of pellet: Briefly, 800 µl of 105,000g crude membrane fraction (6 mg/ml protein) prepared as described under 2.7(b)(i-ii) was centrifuged at 3000 rpm for 20 minutes at 4°C using the Chilspin centrifuge. The supernatant was discarded whilst the pellet was saved.

ii) Pellet solubilisation: The pellet obtained above was resuspended in 100 µl Triton X -100 buffer (1% Triton X -100, 20% glycerol in 0.25 M Tris pH 6.8). The mixture was shaken vigorously for 30 minutes at 26°C, to solubilise the membrane proteins.

iii) Incubation with EGF: The mixture was then centrifuged at 14,000g for 20 minutes on Mini Centaur Centrifuge (MSE) at 4°C. 50 µl of supernatant was incubated in the presence and absence of 60 ng unlabelled EGF in buffer B, so that the final volume was 62 µl, for 30 minutes at 37°C.

iv) Phosphorylation: Incubation tubes were placed on ice and the contents of each tube added to dried-down residue of 10 µCi [$\gamma$-$^{32}$P] ATP. Incubation on ice was continued for a further 5 minutes. The reaction was stopped by adding equal volume, i.e. (62 µl) of Tris glycine electrophoresis buffer (1% SDS, 10% Triton X -100, 15% glycerol, 0.05% bromophenol blue, 5% β - mercaptoethanol in 50 mM Tris, pH 6.8.

v) Electrophoresis: The mixture was boiled for 3 minutes. Aliquots containing 100 µg protein were used for SDS-PAGE.

vi) Autoradiography: After the electrophoresis as described under 2.13(iv-vi), the dried gel was subjected to autoradiography at -70°C for 2 weeks, using hyperfilm TM obtained from Amersham as described under 2.13(vii).

## 2.14 IMMUNOCYTOCHEMISTRY

Two immunocytochemical techniques using monoclonal antibodies to the external and internal domains of the EGF receptor, are described:-

a) Indirect immunoperoxidase technique.

b) Labelled avidin-biotin technique.

### a) Indirect immunoperoxidase technique using a monoclonal antibody to the binding site (external domain) of the EGF receptor:

The method was a modification of the methods of Delellis et al (1979); Van Noorden and Polak (1983).

i) Briefly, 3 - 4 µM cryostat sections were cut and collected on lysine coated slides and allowed to dry with a fan for 30 minutes.

ii) Endogenous peroxidase activity was blocked by immersing sections for 30 minutes in a solution of 0.3% hydrogen peroxide in acetone. Sections were then rinsed in Tris buffer containing 0.9% (w/v)NaCl, pH 7.6 (TBS) and 1% Triton X -100 to decrease background staining. Slides were dried except for the area of the section and placed in damp chamber.

iii) Background blocking was achieved by layering 100 µl normal rabbit serum (NRS) 1/4 dilution in TBS on sections and incubating for 10 minutes at room temperature, after which NRS was gently poured off.

iv 1st layer: 100 µl of a 1/30 dilution of primary antibody raised in mouse, diluted in NRS (1/6 dilution in TBS) was layered on the section. The section was incubated for 30 minutes and rinsed twice with TBS for 5 minutes each. 100 µl of NRS (1/4 dilution in TBS) was layered on the section and incubated for 10 minutes. Excess NRS was gently poured off.

v) 2nd layer: 100 µl of (1/25 dilution) rabbit antimouse peroxidase conjugated immunoglobulin (RAMPC) (2nd antibody) (Dako) was applied on the section and incubated for 30 minutes. At the end of the incubation the section was washed twice for 5 minutes each.

vi) Peroxidase development: The section was incubated in freshly prepared diaminobenzidine (DAB) in Tris buffer (pH 7.6) containing hydrogen peroxide (final concentration 0.04%) for 20 minutes. The experience of the author has shown that prostate sections required up to 20 minutes incubation with DAB to achieve full colour development.

All sections were therefore incubated with DAB for this period of time.

Note: DAB is carcinogenic and therefore must be handled with care.

vii) Counter staining; The section was subsequently counter-stained with Harris haematoxylin (1 - 5%) for up to 5 minutes, and washed in running tap water for 2 minutes.

viii) Mounting: The section was dried at room temperature for up to 1 hour, and mounted in DPX.

ix) Controls: As a negative control NRS was substituted for

the primary antibody. For positive control, prostatic acid phosphatase (PAP) antibody was substituted for the primary antibody to check the technique and reagents.

b) Labelled avidin-biotin method:

The method was a modification of the method of Guerdon et al (1979).

i) Briefly, 3 - 4 µm cryostat sections were cut and collected on lysine coated slides and were allowed to dry.

ii) Sections were fixed in acetone for 20 minutes and rinsed in Tris buffered saline (TBS) for 5 minutes.

iii) Primary antibody to external domain ($EGF\text{-}R_1$) 1/30 dilution in 1/6 dilution of NRS in TBS was applied overnight at $4^{o}C$.

iv) Sections were rinsed 2 x in TBS for 5 minutes each.

v) Sections were then covered with biotinylated sheep antimouse immunoglobulin for 30 minutes at room temperature.

vi) Sections were again rinsed in TBS 2 x for 5 minutes each.

vii) Streptavidin - AP was then applied to sections for 15 - 30 minutes at room temperature.

viii) Again, sections were rinsed 2 x for 5 minutes each in TBS.

ix) Sections were covered with fresh alkaline phosphate substrate (Fast red 1 TR, Levamisole, Naphthol ASB1 phosphate) for 20 minutes, after which sections were washed in tap water.

x) Counterstaining was achieved by using Mayers haematoxylin for 1 minute. They were then washed and differentiated in 0.25% aqueous HCl.

xi) After blueing the sections in lithium carbonate solution, they were finally washed and soaked in 0.1% Triton X -100.

xii) Sections were thereafter drained and mounted in glycerine jelly.

## 2.15 EGF RECEPTOR LEVELS IN BPH AND CaP. (TECHNIQUES USED IN MEASUREMENT)

a) Radioligand exchange assay.

b) Indirect immunoperoxidase method:-

i) using monoclonal antibody to the external domain of the EGF receptor (EGF-$R_1$).

ii) using monoclonal antibody to the internal domain of the EGF receptor (F4).

c) Labelled avidin biotin technique:-

i) using monoclonal antibody to the external domain of the EGF receptor (EGF-$R_1$).

ii) using monoclonal antibody to the internal domain of the EGF receptor (F4).

a) Radioligand exchange assay: The receptor binding assay was essentially the same as described under Section 2.11 with minor modification. 100 µl of 8nmol/L $^{125}$I-EGF was incubated with 100 µl of prostate particulate fraction for 90 minutes at 37°C. After PEG precipitation of the bound complex radioactivity retained in the pellet was measured, specific binding was calculated as described under Section 2.11, i.e. by subtracting non-specific from total binding. The mean number of binding sites obtained for both the BPH and CaP tissues were calculated. Also calculated were the receptor levels expressed according to the histologic classification of the tumour based on Gleason score.

b) Indirect immunoperoxidase method using monoclonal antibody to EGF receptor binding site (external domain)

i) This is identical to the method described under Section 2.14 employing DAB. Briefly, 3 - 4 µm thick frozen sections were transferred to microscope slides coated with lysine and fixed in acetone. Sections were subsequently incubated at room temperature with murine monoclonal antibody (EGF-$R_1$) at a dilution of 1 : 30 (v/v) for 30 minutes, followed by a peroxidase conjugate of rabbit antimouse immunoglobulin. Colour was developed with diaminobenzidine made in Tris-HCl saline pH 7.6 containing 0.04% hydrogen peroxide. Sections were finally counterstained with 5% haematoxylin for 2 minutes, dried and mounted using DPX. Normal rabbit serum (NRS) replaced the primary antibody and served as negative control.

The intensity of the staining of each prostate section was assessed by 3 independent investigators using reference slides. The scores for staining ranged from 1 + (weak staining) to 3 + (intense staining). 1 - represents a negative reaction. Staining characteristics were noted for BPH and CaP and also for the various grades by histologic classification according to Gleason score.

Demonstration of internal domain of the EGF receptor (F4):

ii) (Use of monoclonal antibody F4):

F4 is a monoclonal antibody to the intracellular domain of the EGF receptor raised against peptide 2E having the sequence of residues 985 - 996 of the EGF receptor (Gullick et al, 1986; Berger et al, 1987).

Immunoperoxidase technique: The technique was identical to the one described under section 2.14(a). Briefly, 3 - 4 μm frozen cryostat sections of BPH, well differentiated and poorly differentiated tissues were passed through the same staining steps as described under section 2.14(a) but using monoclonal F4 at concentration of 0.05 mg/ml as the primary antibody.

The sections were finally mounted in DPX.

c) Labelled avidin biotin technique:

i) and ii) Use of monoclonal antibodies F4 and EGF-$R_1$ (Monoclonal antibodies to the internal and external domain of the EGF receptor)

The method was based on the technique of Guesdon et al (1979) as described under section 2.14(b). Briefly, frozen sections were treated with primary antibody EGF-$R_1$ (1/30 dilution) or F4 (0.05 mg/ml concentration) and incubated overnight.

Sections were further treated with biotinylated sheep anti-mouse immunoglobulin followed by streptavidin - AP and alkaline phosphatase substrate (Naphthol ASB1 phosphate). Colour was developed by fast red ITR. Sections were counterstained with Mayers haematoxylin and finally mounted in glycerine jelly.

The above procedure was repeated but using F4 instead of EGF-$R_1$.

2.16 OTHER EXPERIMENTS

Experiments designed to test the EGF receptor blocking in BPH:-

a) Receptor site blocking with monoclonal antibody to the EGF receptor sites (EGF-$R_1$).

b) Receptor site blocking with EGF.

Experiments designed to block EGF receptor sites in BPH

Two methods were employed (1) radioligand exchange assay and (2) immunocytochemistry.

a) Radioligand exchange assay: A reaction tube containing particulate fraction suspension was pretreated with buffer B and used as control.

Another tube also containing particulate suspension was pretreated with monoclonal antibody (1/30 dilution v/v) to EGF receptor site.

After incubation at room temperature for 30 minutes, the tubes were centrifuged at 3000 rpm for 20 minutes. The supernatants were discarded. The pellets were thereafter taken up with 100 µl buffer B. 100 µl $^{125}$I-EGF 8.0 nmol/L was added and receptor binding assay was carried out in the presence and absence of 50-fold excess unlabelled EGF, as described under section 2.11(a). Specific binding in the control sample was compared with the test sample.

b) Immunocytochemistry: A cryostat section of BPH was pretreated with TBS and used as control. Another section was pretreated with 300 nmol/L unlabelled EGF.

After incubation for 30 minutes at room temperature, the sections were rinsed with Tris buffered saline (TBS). 100 µl of primary antibody (EGF-$R_1$) was applied to the sections and were subjected to all the steps in immunocytochemistry as described under section 2.14(a)(i-x).

Staining of the test slide was compared with that of the control.

## 2.17 STATISTICAL ANALYSIS

a) All experimental analyses were carried out in duplicate and mean values used.

b) Coefficient of variation (CV) values of between and within batches were calculated.

c) Student's 't' test as described by Swinscow (1982) was used to analyse statistical differences between means of receptor levels in BPH and CaP and also between means of receptor levels in well differentiated CaP and poorly differentiated CaP.

# CHAPTER 3

# RESULTS

## CHAPTER 3. RESULTS

In this chapter results will be presented as follows:

3.1 Separation methods

3.2 Validation of EGF receptor assay

3.3 Characterisation of EGF receptor

3.4 Molecular characterisation of the EGF receptor

3.5 i) Immunocytochemical demonstration of the presence of the EGF receptor

ii) Comparison between radioligand assay and immunocytochemistry

3.6 Demonstration of the presence of EGF receptors in CaP

3.7 EGF receptor levels in BPH and CaP compared

3.8 Demonstration of the presence of internal domain of the EGF receptor

3.9 Other experiments: EGF receptor blocking experiments

3.1 Separation of bound complex from free:

Filtration and centrifugation are the two main techniques used by many workers to separate specific from non-specific binding when radioligand assay technique is used, but no-one has established which of the two techniques is more suitable to apply when using radioligand assay technique on human BPH. In view of this, steps were taken to optimise and compare the two techniques of filtration and centrifugation.

The following observations were made on the experiments undertaken:-

a) Filtration and washing effect: As indicated by Figure 11, two washes of the radioactivity retained on the filter paper were optimal. Beyond the two washes specific binding started to fall. This showed that the number of washes of the retained radioactivity on the filter paper has important implications on the receptor levels in the human BPH tissue.

b) Filtration and centrifugation compared: In order to improve upon the separation technique the method of Hwang et al (1986), which involved polyethylene glycol (PEG) precipitation prior to centrifugation, was adopted and compared with filtration technique. Figure 12 showed that the PEG precipitation and centrifugation was superior to the filtration technique, because the PEG method produced 25% more specific binding than what was achieved by filtration method. Furthermore, the background count for the PEG method was 26% lower than the filtration technique, whilst the levels of non-specific binding were identical in both cases.

c) Aspiration compared with pouring of supernatant: Given the above encouraging results produced by the PEG method, it was decided to identify and optimise factors that might improve the PEG method further. In this regard, after PEG precipitation and centrifugation,

Figure 11.

Effect of filtration and washing on EGF binding

$^{125}$I-EGF in the $10^{-9}$ concentration range was incubated at 37°C for 40 mins with particulate fraction of BPH as described under section 2.9. At the end of the incubation period the bound complex was separated from the free $^{125}$I-EGF using filtration technique as described under section 2.9.

Briefly, the bound complex was filtered through a microfibre filter under reduced pressure. The radioactivity retained by the filter was either washed once, twice, thrice or four times in separate experiments with 1 ml cold buffer B (4°C) as described under 2.9. The final washed pellets on the filter paper were counted for specific binding.

The values were means of 3 different samples, each analysed in duplicate.

The relationship between specific binding and the number of washes was established.

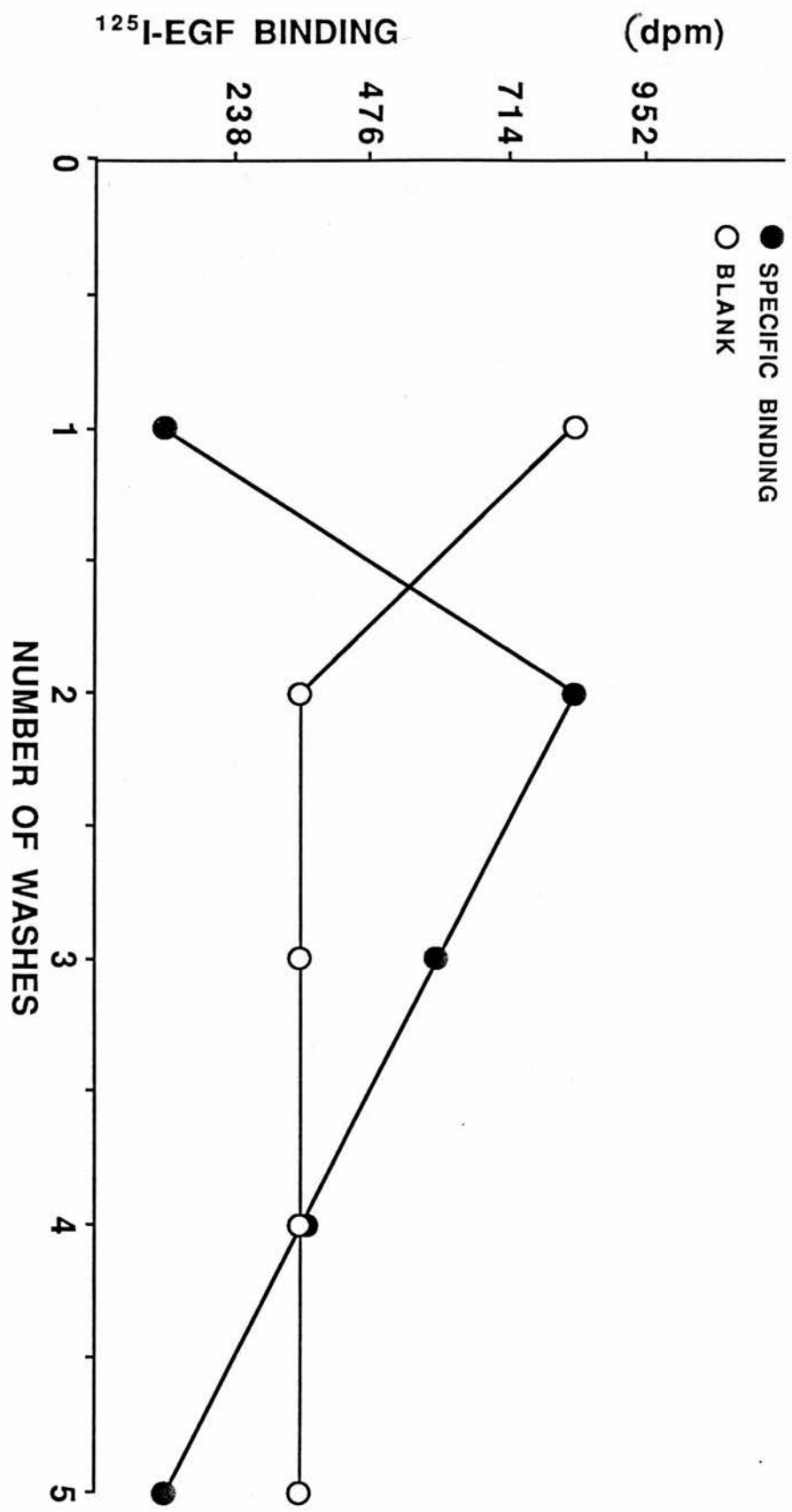

$^{125}$I-EGF BINDING (dpm)
238
476
714
952
0
1
2
3
4
5
NUMBER OF WASHES
SPECIFIC BINDING
BLANK

Figure 11.

Figure 12.

$^{125}$I-EGF binding: Filtration and polyethylene glycol (PEG) separation methods compared

100 µl $^{125}$I-EGF and 100 µl BPH particulate fraction were incubated in the presence and absence of 50-fold excess unlabelled EGF in 200 µl buffer B for 40 minutes at 24°C.

Bound $^{125}$I-EGF complex was separated from free $^{125}$I-EGF either by (A) filtration under reduced pressure or (B) by PEG precipitation and centrifugation.

The filtration technique has been described under section 2.9 but, briefly, after incubation the mixture was filtered through Whatman microfibre filters (Whatman GF/A) under reduced pressure. The radioactivity retained on the filter paper was washed and counted.

The PEG method is also described under section 2.11 but, briefly, after incubation 1 ml of 20% PEG and 0.1% BSA was added to the mixture. After 10 minutes, the mixture was centrifuged for 20 minutes at 3000 rpm. The supernatant was discarded and the pellet washed with 10% PEG and 0.05% BSA and the process of centrifugation repeated.

In each of the two experiments the radioactivity left in the microfibre filter or in the pellet was counted and specific binding calculated as in section 2.9. The two results (A and B) were compared.

Values were means of 3 different samples, each analysed in duplicate.

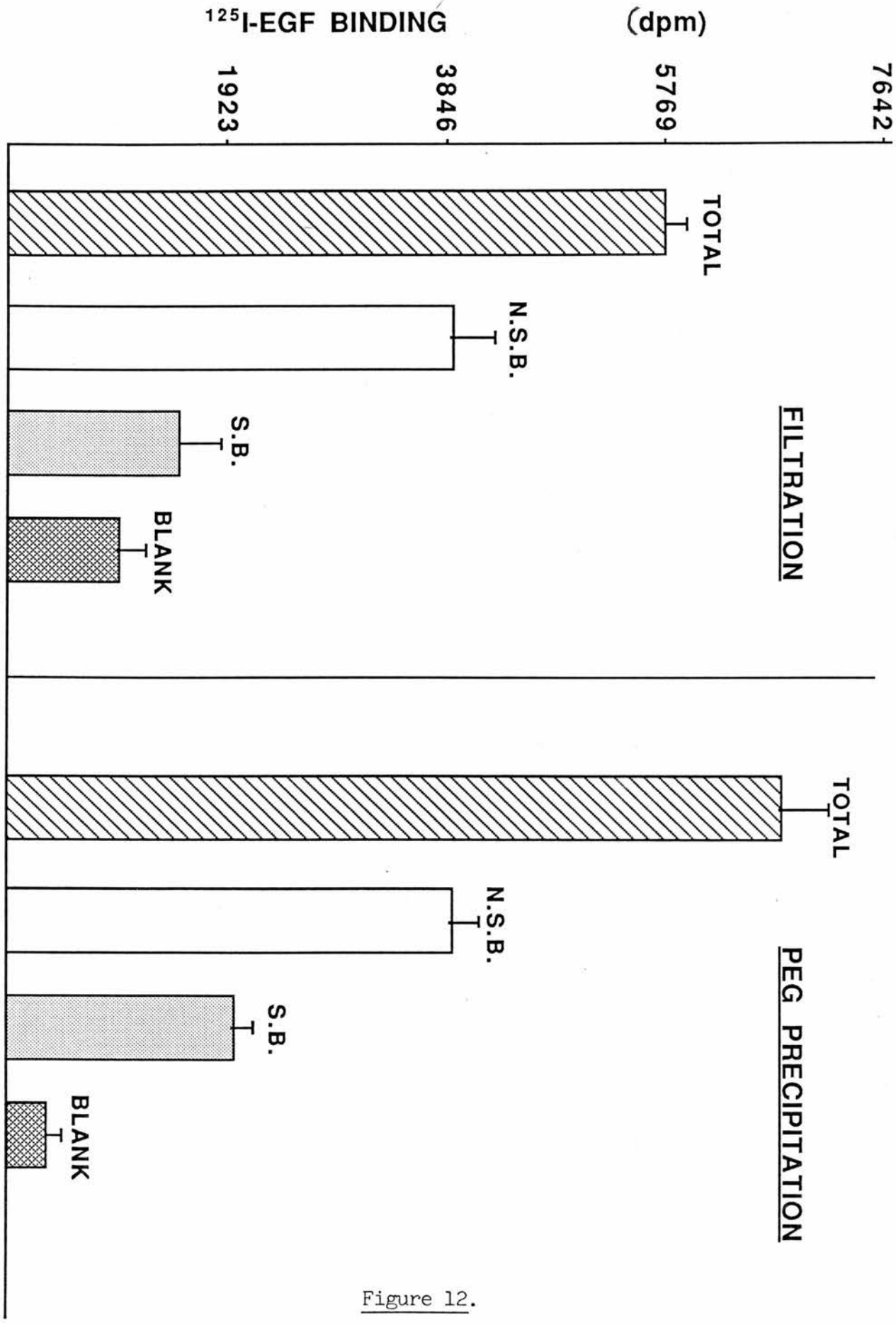

$^{125}$I-EGF BINDING (dpm)
1923
3846
5769
7642
TOTAL
N.S.B.
S.B.
BLANK
FILTRATION
TOTAL
N.S.B.
S.B.
BLANK
PEG PRECIPITATION
NSB = NON SPECIFIC BINDING
SB = SPECIFIC BINDING

Figure 12.

aspiration of supernatant was compared with pouring off the supernatant. Figure 13 showed that aspiration of the supernatant produced a much more consistent result, but the pouring method produced about 12.5% more specific binding than the aspiration technique. The pouring method, however, had a drawback of producing very high background count. PEG precipitation and centrifugation followed by aspiration was therefore selected as the most reliable and reproducible method of separation for the human BPH.

d) Impact of centrifugation: In order to precipitate the bound complex more efficiently after the addition of PEG solution, the incubation medium was subjected to various centrifugation times to find the impact of centrifugation on the level of EGF specific binding. Figure 14 shows that centrifugation at 3000 rpm for 5 minutes as practised by other workers (Hwang et al, 1986), grossly underestimated the specific binding.

Centrifugation for 30 minutes produced the maximum specific binding (mean $\pm$ SD = 800$\pm$ 50 dpm), but this was also accompanied by the highest non-specific binding. On the other hand centrifugation for 20 minutes produced specific binding (mean $\pm$ SD = 750$\pm$ 45 dpm) which was not statistically different from what was obtained for 30 minutes centrifugation. Furthermore, centrifugation for 20 minutes was accompanied by the lowest non-specific binding. Above all, the specific binding after 20 minutes centrifugation was observed to be the most reproducible. The level of specific binding started to decline after 30 minutes centrifugation. 20 minutes centrifugation was therefore adopted for use in all subsequent assays.

Figure 13.

Aspiration compared with pouring of supernatant

Receptor binding assay, PEG precipitation and centrifugation were carried out as described under 2.11.

Briefly, at the end of the incubation period, the bound complex was precipitated by 20% PEG and 0.1% BSA and centrifuged. In one set of experiments the supernatant was poured off (A). In another set, the supernatant was aspirated off using venturi pump which provided a negative pressure (B).

The process was repeated after washing the pellet once with 10% PEG and 0.05% BSA. Bound radioactivity retained by each method was measured on a gamma counter. Levels of specific binding achieved by both techniques (A and B) were compared.

Values were means of 4 different samples, each analysed in duplicate.

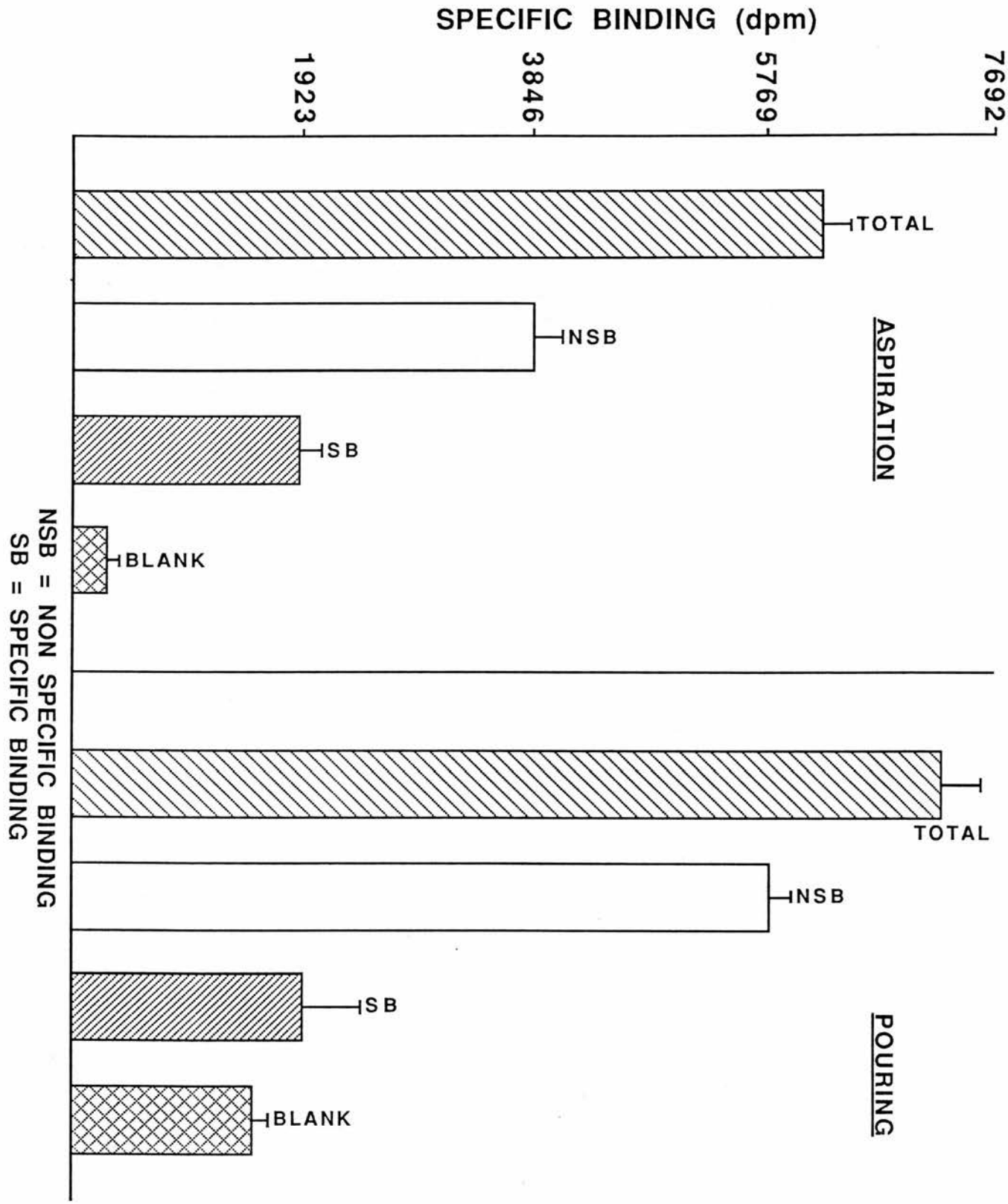

SPECIFIC BINDING (dpm)
1923
3846
5769
7692
TOTAL
NSB
SB
BLANK
ASPIRATION
TOTAL
NSB
SB
BLANK
POURING
NSB = NON SPECIFIC BINDING
SB = SPECIFIC BINDING

Figure 13.

Figure 14.

PEG precipitation: Impact of centrifugation time on binding

The receptor binding assay was as described under section 2.11.

Briefly, after the precipitation of the $^{125}$I-EGF bound complex with PEG, the mixture was centrifuged for 5, 10, 20, 30 and 45 minutes respectively at 3000 rpm. After washing the pellet with 10% PEG in 0.05% BSA as described under section 2.11, the centrifugation procedure was repeated. The pellet obtained after each period of centrifugation was counted on a gamma counter. The specific binding obtained for the various centrifugation periods were compared.

Values are means of 3 different samples, each analysed in duplicate.

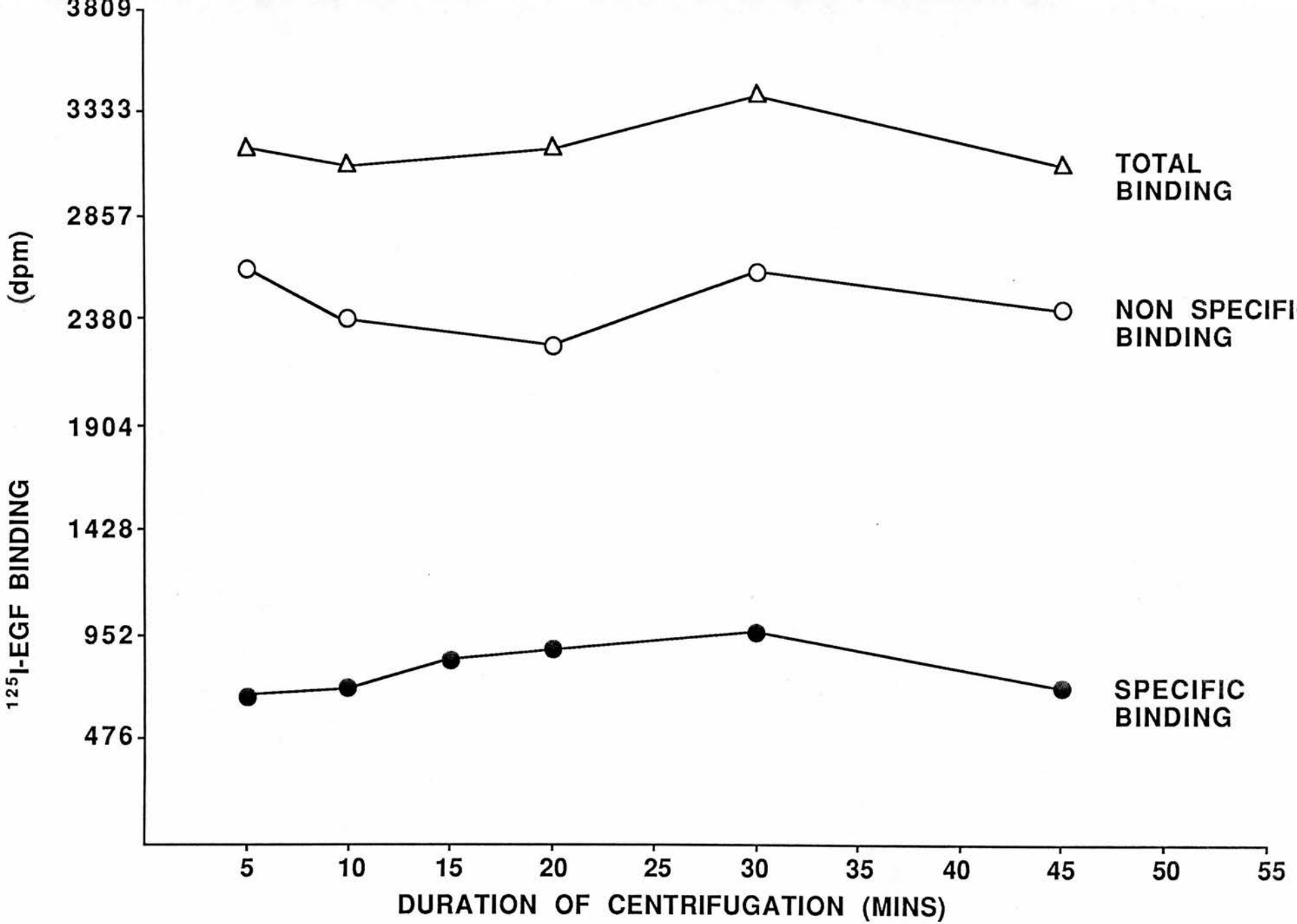

3809
3333
2857
2380
1904
1428
952
476
(dpm)
$^{125}$I-EGF BINDING
TOTAL BINDING
NON SPECIFIC BINDING
SPECIFIC BINDING
5
10
15
20
25
30
35
40
45
50
55
DURATION OF CENTRIFUGATION (MINS)

Figure 14.

3.2 Validation of the EGF receptor assay:

a) The assay was validated using the human placenta, because it was known to contain high levels of EGF receptors (Hock and Hollenberg, 1980; Ramani et al, 1986).

The outcome of the validation experiments have been described under section 2.11(a). These showed that the receptors were saturable, specific and of high affinity. Scatchard plot analysis revealed one class of binding site consistent with observation made by Hock + Hollenberg (1980), although Ramani et al (1986) observed two classes of binding sites. The rest of the characteristics observed in this report were identical to those made by Hock + Hollenberg (1980) and Ramani et al (1986). This showed that EGF was bound to its own classical receptor.

b) In another set of experiments, binding between $^{125}$I-EGF and human plasma was investigated. The outcome of this investigation is described under 2.11(b). The absence of binding observed shows that the binding in the placenta is not due to contamination from human blood. This lends further support to the specificity of the binding seen in the placenta.

3.3 Characterisation of the EGF receptor:

The EGF receptor was characterised under the following headings:-

a) Distribution of the EGF receptors in the subcellular fractions of BPH

b) Effect of protein concentration on EGF binding

c) Effect of time and temperature on EGF binding

d) Dissociation of EGF bound complex

e) Saturation studies

f) Scatchard plot analysis

g) Competition studies

h) Impact of pH on binding

i) Thermal sensitivity

j) Effect of enzyme and enzyme inhibitors

k) Effect of $MgCl_2$ and DCC on binding

l) Effect of storage at $-70^{o}C$ on binding

m) Binding of urogastrone and EGF by BPH

The results of these investigations are now presented.

Characterisation of the EGF receptor in the human BPH tissue

EGF receptors have been characterised in the rat prostate by various biochemical techniques (Traish+Wotiz, 1987) but earlier attempts to characterise the receptor in the human BPH tissue using biochemical method were not successful (Gregory et al, 1986). This was one of the reasons which prompted the author to localise and characterise the EGF receptor in the human BPH, using a whole range of biochemical techniques.

The outcome of the characterisation are described as follows:-

a) Subcellular distribution of the EGF receptors in the human prostate:

In order to investigate the distribution of the EGF receptors in the human prostate tissue, the homogenate preparation was subjected to subcellular fractionation as outlined under section 2.12(a). Each fraction was subjected to $^{125}$I-EGF binding assay. TABLE 3 shows that over 68% of the specific binding was associated with the 800g (crude heavy pellet) fraction. The remainder of the binding was distributed between the microsomal pellet (105,000g) and the mitochondrial pellet (15,000g). No specific binding was observed with the 105,000g supernatant (the cytosol). In view of the low level of binding associated with the microsomal fraction, it was decided to centrifuge the homogenate at 105,000g to obtain a total particulate fraction (pellet) containing all the receptors.

b) Effect of protein concentrations on receptor estimations:

In order to determine the limit to which tissue homogenates must be diluted with buffer for accurate receptor estimations, total particulate fraction pellets were resuspended in different volumes of buffer to provide a wide range of protein concentrations, as described under section 2.12(b). Figure 15(A) shows that EGF binding is linear with dilutions corresponding to protein concentrations between 0.1 and 1 mg/ml. Beyond 1 mg/ml protein binding appears to decline. A second linearity was, however, observed between 1.5 and 8 mg/ml (Figure 15(B)). The appearance of this second linearity is not understood. The specific binding achieved at 8 mg/ml was only 30% above that of 1 mg/ml.

c) Effect of time and temperature on $^{125}$I-EGF binding:

Figure 16 illustrates the patterns of binding of EGF to the 105,000g particulate pellet at three temperatures. Clearly the

TABLE 3

a) SUBCELLULAR DISTRIBUTION OF RECEPTORS FOR EGF IN THE HUMAN PROSTATE

Subcellular fractions were prepared according to the method of Leake, Chisholm and Habib (1983) as described under section 2.7(c).

Briefly, particulate fraction was centrifuged at 800g to obtain the crude heavy pellet. The supernatant was centrifuged at 15,000g to obtain the mitochondrial pellet. The supernatant was again centrifuged at 105,000g to obtain the microsomal pellet, using the Sorvall ultra-centrifuge as described under section 2.7(c). Pellets were each reconstituted in 1 ml of buffer B, and aliquots were used in binding studies as described under section 2.12(a). Briefly, 100 µl $^{125}$I-EGF and 100 µl particulate fractions were incubated in the presence and absence of 200 ul 50-fold excess unlabelled EGF for 40 minutes at 24$^{o}$C. After incubation $^{125}$I-EGF bound complex was separated from free $^{125}$I-EGF by PEG precipitation and centrifugation. Specific binding was subsequently calculated by the difference between total and non-specific binding.

The experiment was performed on 3 different samples and values are expressed as means SD.

TABLE 3

| Fraction | Specific Binding (dpm/100 µl) | % Bound* |
|---|---|---|
| 800g crude pellet | 1182 ± 173 | 68 ± 4 |
| 15000g pellet (Mitochondria) | 365 ± 53 | 21 ± 5 |
| 105000g pellet (Microsome) | 188 ± 25 | 10 ± 1 |
| 105000g supernatant (Cytosol) | 0 | 0 |

*% of total binding

Figure 15.

Impact of protein concentration on EGF receptor estimation

100 µl aliquots of BPH particulate suspension containing 0.1 to 8 mg protein per ml were incubated with 100 µl aliquots of $^{125}$I-EGF (8.0 nmol/L) in the presence and absence of 50-fold excess unlabelled EGF for 40 minutes at 24$^{o}$C. At the end of the incubation $^{125}$I-EGF bound was separated from free by PEG precipitation and centrifugation as described under section 2.12(b). Specific binding was calculated by subtraction of non-specific from total.

Values are means of 4 different specimens, each analysed in duplicate.

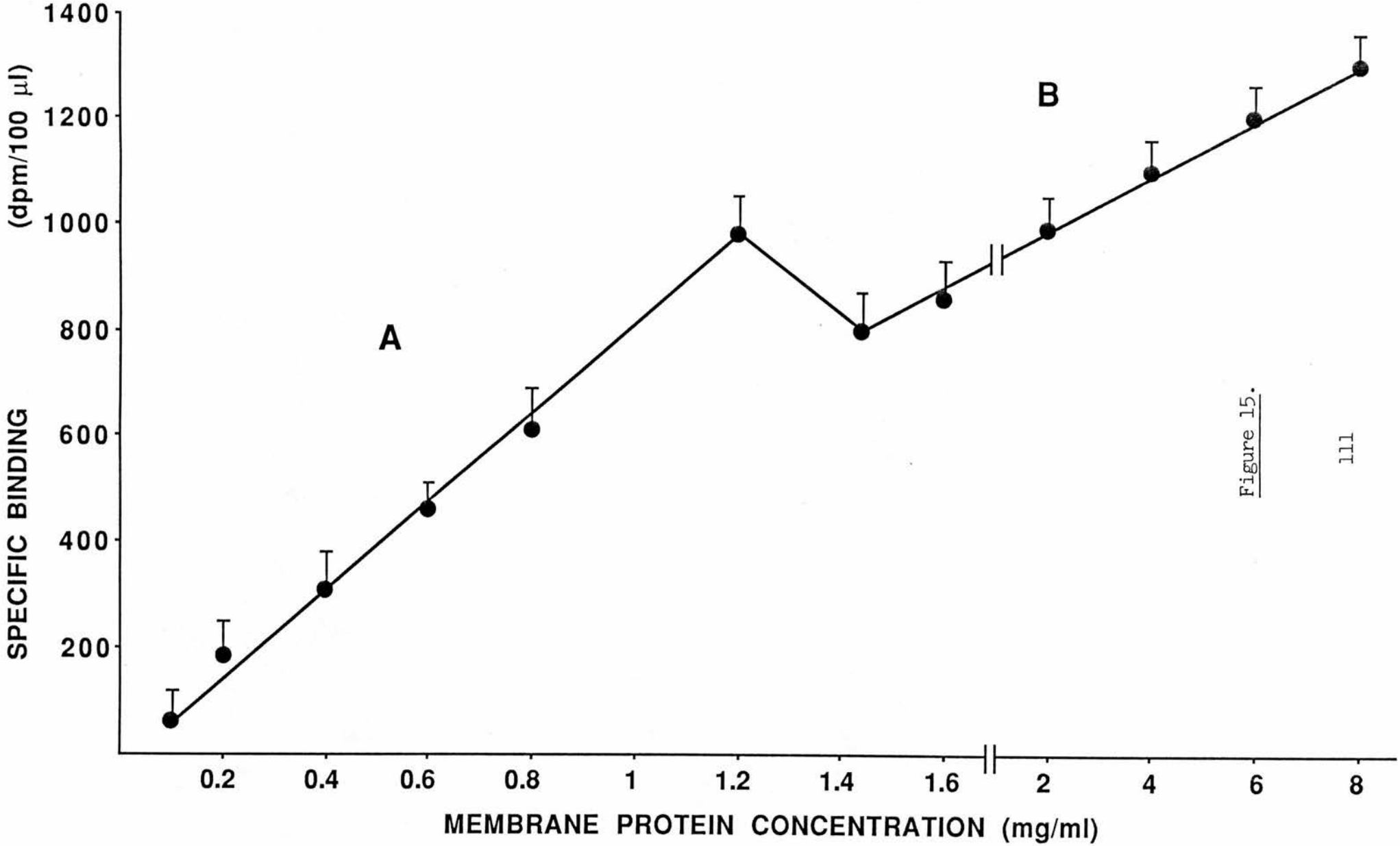

1400
1200
1000
800
600
400
200
(dpm/100 µl)
SPECIFIC BINDING
A
B
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
2
4
6
8
MEMBRANE PROTEIN CONCENTRATION (mg/ml)

Figure 15.

Figure 16.

Effect of time and temperature on $^{125}$I-EGF binding

100 µl BPH particulate fraction containing 1 mg/ml protein was incubated with 100 µl 8.0 nM $^{125}$I-EGF in the presence and absence of 50-fold excess unlabelled EGF (in 200 ul buffer B) for up to 90 minutes at 4°C, 25°C and 37°C.

At the end of the incubation period, $^{125}$I-EGF bound complex was separated from free $^{125}$I-EGF by PEG precipitation and centrifugation as described under section 2.11.

Specific binding was calculated by a difference between total and non-specific binding after counting on gamma counter.

Binding profiles obtained for each temperature are illustrated.

The values are means of 3 different samples, each analysed in duplicate.

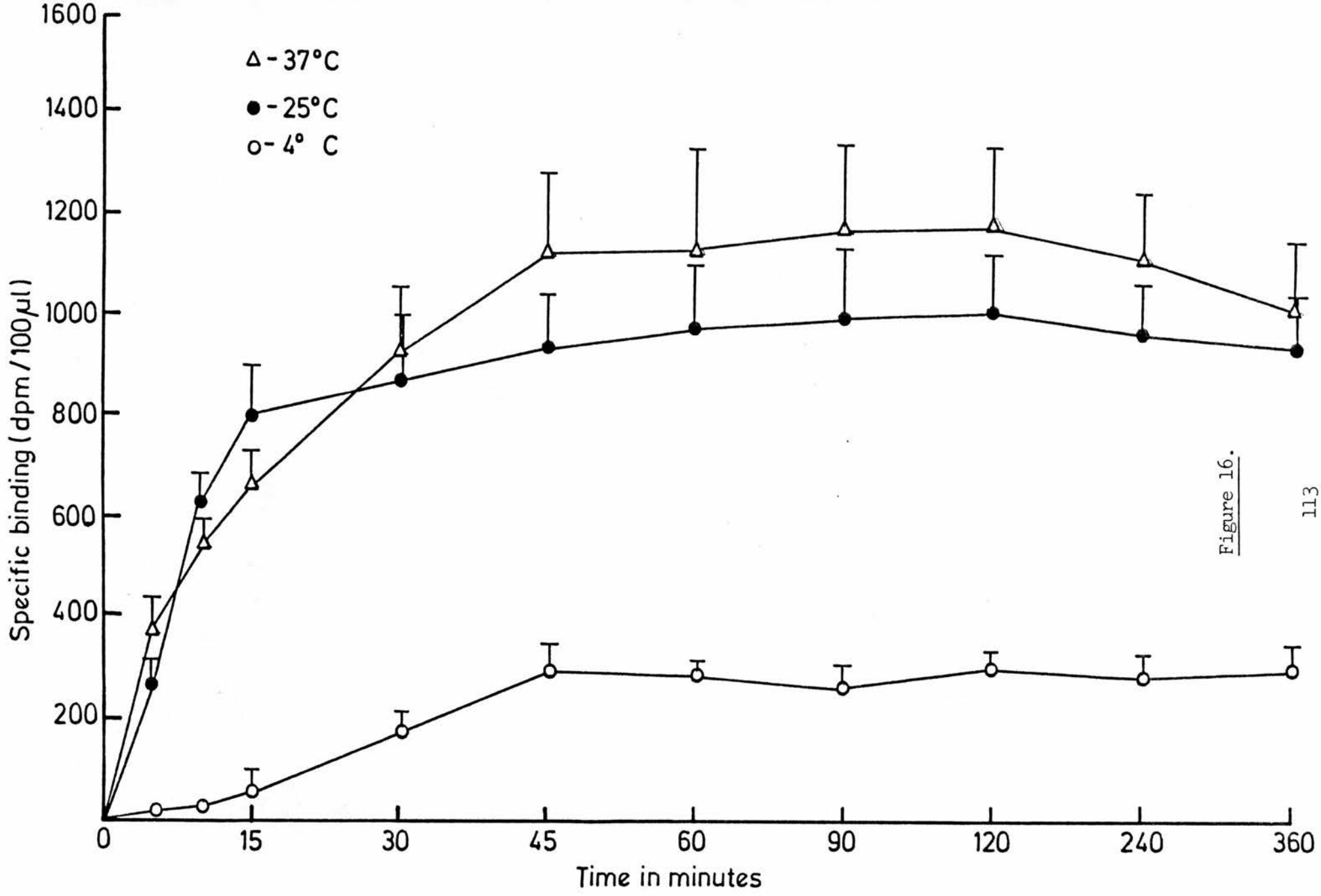

Δ - 37°C
● - 25°C
o - 4° C
Specific binding (dpm / 100µl)
1600
1400
1200
1000
800
600
400
200
0
15
30
45
60
90
120
240
360
Time in minutes

Figure 16.

binding was time and temperature dependent, with maximal binding obtained at 37°C after 45 minutes of incubation. The maximal binding was sustained for a further 75 minutes beyond which a decline was observed, probably due to the instability of the particulate fraction under these conditions. Although the binding at 25°C was slightly lower than that observed at 37°C, the binding profiles for both temperatures were similar whereas at 4°C receptor-ligand interaction was considerably slower and significantly reduced. It was therefore decided to undertake all subsequent incubations at 37°C for 90 minutes.

d) Dissociation of BPH particulate fraction bound $^{125}$I-EGF in the presence of 50-fold excess unlabelled EGF

It has been observed that association of $^{125}$I-EGF with BPH particulate fraction was time and temperature dependent (Figure 16). In order to demonstrate if the dissociation of the $^{125}$I-EGF bound complex was also time dependent and also to find the influence of presence of unlabelled EGF on the dissociation, this investigation was carried out.

Figure 17 (A and B) is showing dissociation of $^{125}$I-EGF bound complex in the absence (A) and presence (B) of 50-fold excess unlabelled EGF. The bound iodinated EGF complex was found to be partially dissociated in the presence and absence of excess unlabelled EGF as a function of time at 37°C. In the presence of excess unlabelled EGF, the dissociation was considerably enhanced. These observations indicated that both the association of $^{125}$I-EGF with BPH and the dissociation of $^{125}$I-EGF bound complex were time dependent.

e) Saturation studies: Adamson + Rees (1981) expressed the view that EGF may be considered as a hormone as well as a growth factor. It is also a widely held view that in order to conform to the definition

Figure 17.

Dissociation of particulate fraction $^{125}$I-EGF bound complex in the presence and absence of unlabelled EGF

The method is described under section 2.11. Briefly, after EGF receptor binding assay, as described under section 2.11, the unbound $^{125}$I-EGF was removed by centrifugation. The pellets containing bound complex were resuspended in buffer B containing 50-fold excess unlabelled EGF (B) or no unlabelled EGF (A). Samples were reincubated for indicated lengths of time, i.e. 0 - 180 minutes at 37$^{o}$C.

Controls were precipitated with PEG immediately and centrifuged to separate bound from free $^{125}$I-EGF. This represented 100% binding.

Specific binding for each time was calculated by the difference between non-specific and total binding.

Dissociation took place in the absence of unlabelled EGF (A) and in the presence of unlabelled EGF (B).

The experiment was performed on 3 different samples. Mean values of results are expressed as % of control.

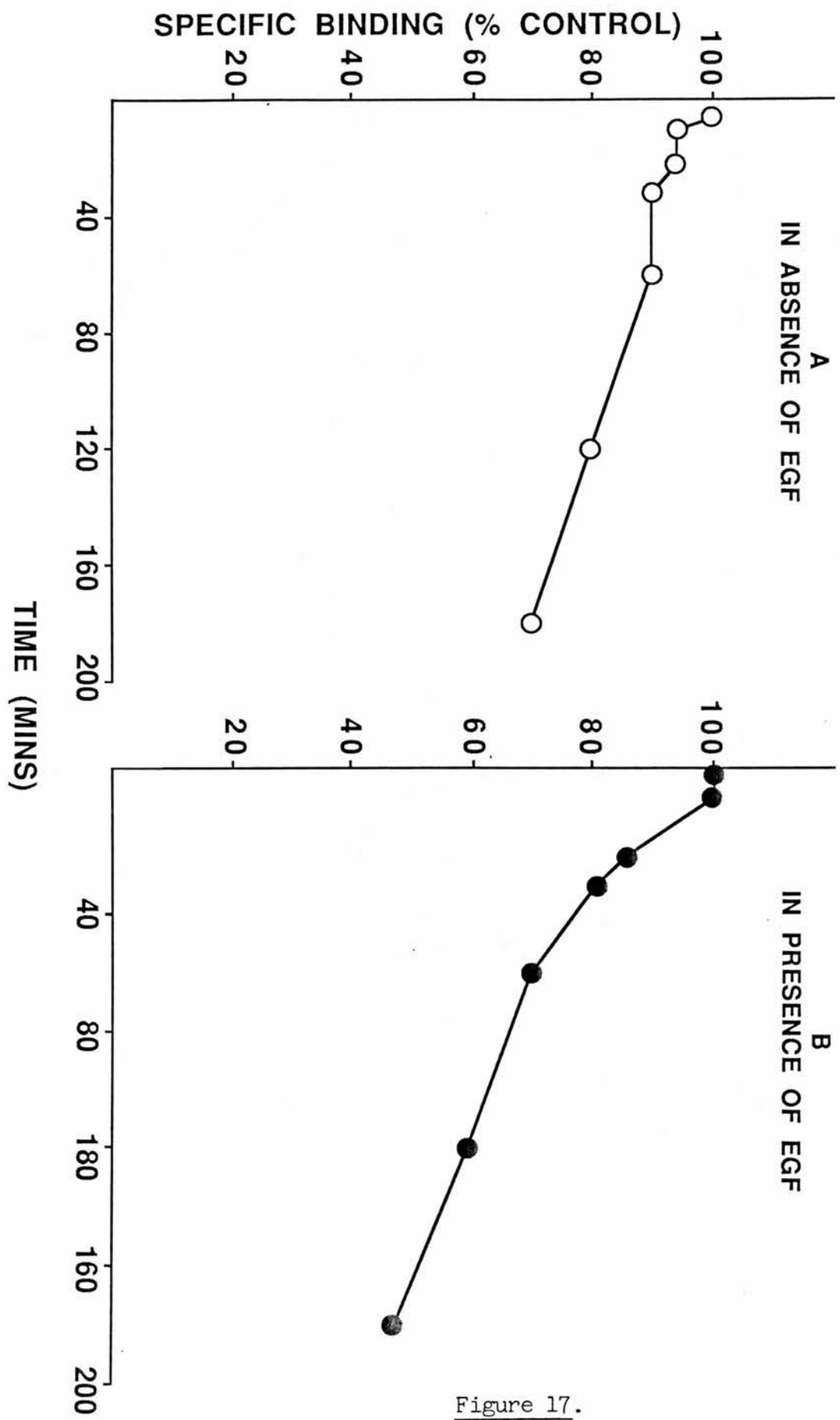

SPECIFIC BINDING (% CONTROL)
20
40
60
80
100
A
IN ABSENCE OF EGF
B
IN PRESENCE OF EGF
40
80
120
160
200
180
TIME (MINS)

Figure 17.

of a hormone, a molecule whether it is a polypeptide or steroid in nature must undergo a specific high affinity saturable interaction with a receptor in the target tissue.

In order to investigate the saturability of the EGF receptor sites, saturation studies as outlined under section 2.12(e) were undertaken.

Figure 18 (insert) reveals that EGF receptors in the human prostate are saturable at $^{125}$I-EGF concentrations above 8 nmol/L when binding is observed to form a plateau. This finding is in agreement with the report of Mukku+Stancel (1985) who found that saturation of rat uterus EGF receptors occurred between 5 - 8 nmol/L EGF concentrations.

f) Scatchard analysis: Given that the receptor sites were saturable, it was decided to investigate the number of binding sites and the affinity of the receptors by Scatchard plot analysis (Scatchard, 1949), using the data obtained from the saturation studies carried out on ten separate prostates. Figure 18 reveals that there are two classes of binding sites. The first of higher affinity (mean Kd = 0.8 $\pm$ SD 0.2 nmol/L) and the second lower affinity (mean Kd = 7.6 $\pm$ SD 2.8 nmol/L). The mean binding capacities for the two components are 14 $\pm$ 1.4 fmol/mg protein and 137 $\pm$ 23 fmol per mg protein respectively.

g) Competition studies: Given that the EGF receptor in the prostate was saturable and of high affinity, it was left to demonstrate if the binding between the growth factor and the receptor was specific.

To do this, the approach outlined in section 2.12(g) was adopted. Figure 19 demonstrates that increasing concentrations of unlabelled EGF inhibited $^{125}$I-EGF binding to BPH particulate fraction, in a dose dependent manner. In the same experiment unlabelled human insulin,

Figure 18

Saturation Studies

The method is described under section 2.12(e).

Briefly, the receptor binding assay was performed using $^{125}$I-EGF over a range of 0.5 nMol - 24 nMol/L and BPH particulate fraction containing 1 mg/ml protein in the presence and absence of 50-fold excess unlabelled EGF at each concentration of $^{125}$I-EGF. After incubation for 90 minutes at 37$^{o}$C, $^{125}$I-EGF bound complexes were separated from free by PEG precipitation and centrifugation as described under section 2.12(e).

Specific binding was obtained by subtraction of non-specific from total binding.

Values are means of 3 different samples, each analysed in duplicate.

Figure 18

Scatchard Analysis

Data obtained from the saturation studies detailed under section 2.12(e) was used in calculating the dissociation constant (kd) and estimating the number of binding sites of the receptor protein by the method of Scatchard (1949).

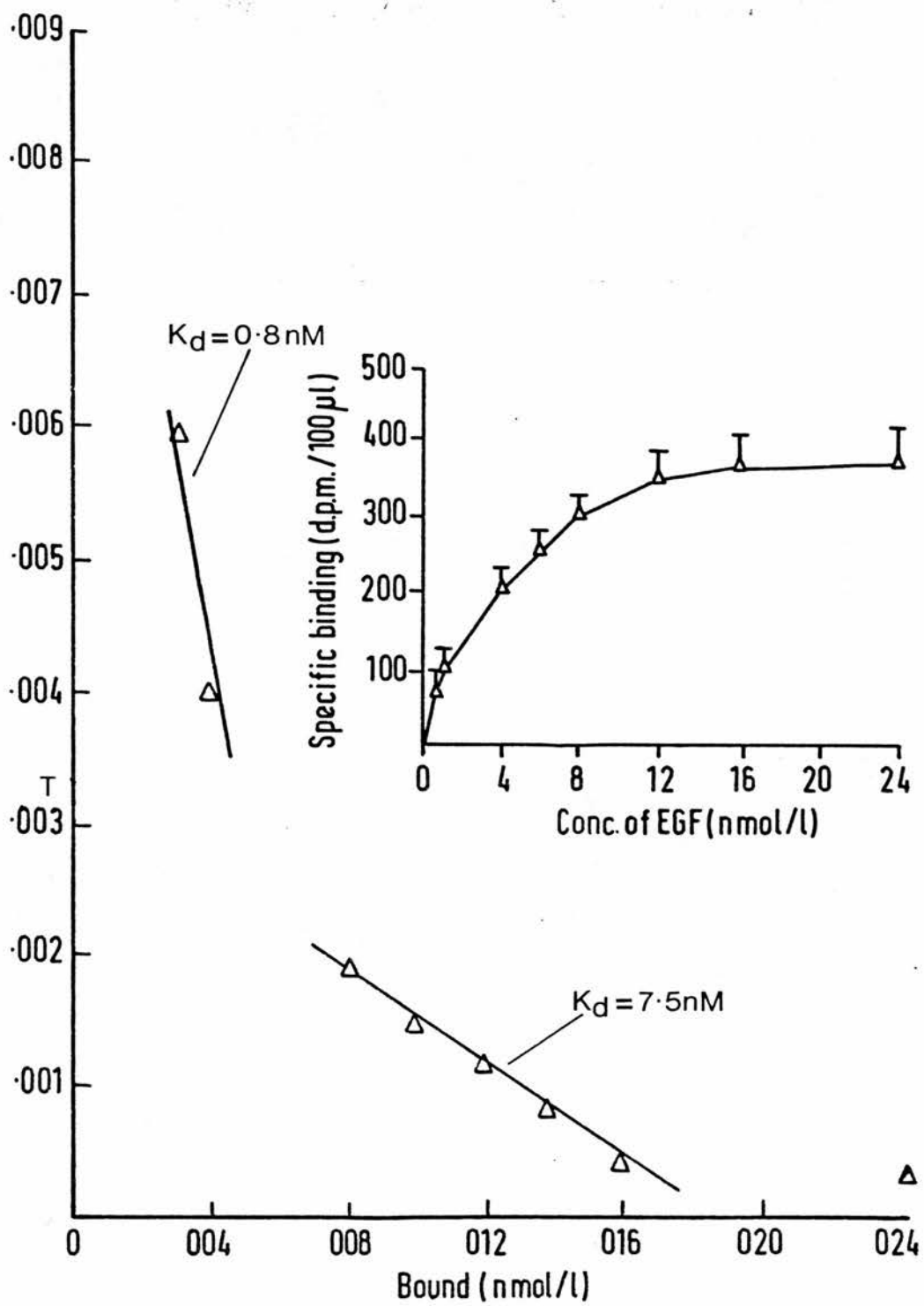

·009
·008
·007
·006
·005
·004
T
·003
·002
·001
$K_d = 0{\cdot}8\,nM$
$K_d = 7{\cdot}5nM$
Specific binding (d.p.m./100µl)
500
400
300
200
100
0
4
8
12
16
20
24
Conc. of EGF (nmol/l)
0
004
008
012
016
020
024
Bound (nmol/l)

Figure 18.

Figure 19.

Competition studies

The method is described in section 2.12(g).

Briefly, 100 µl $^{125}$I-EGF was incubated with 100 µl BPH particulate fraction in the presence and absence of 250 - 3000 ng/ml of unlabelled m-EGF, h-GH, h-FSH, h-LH, human PRL, human insulin and venom nerve growth factor at 37°C for 90 minutes.

At the end of the incubation period, $^{125}$I-EGF bound complex was separated by PEG precipitation and centrifugation as described under section 2.12(g).

The displacement achieved by each competition is noted.

The experiment was repeated on 3 different samples. Results are means of 3 samples, but are expressed as a % of control.

Control sample was not incubated with any competitor, and therefore represents 100% binding.

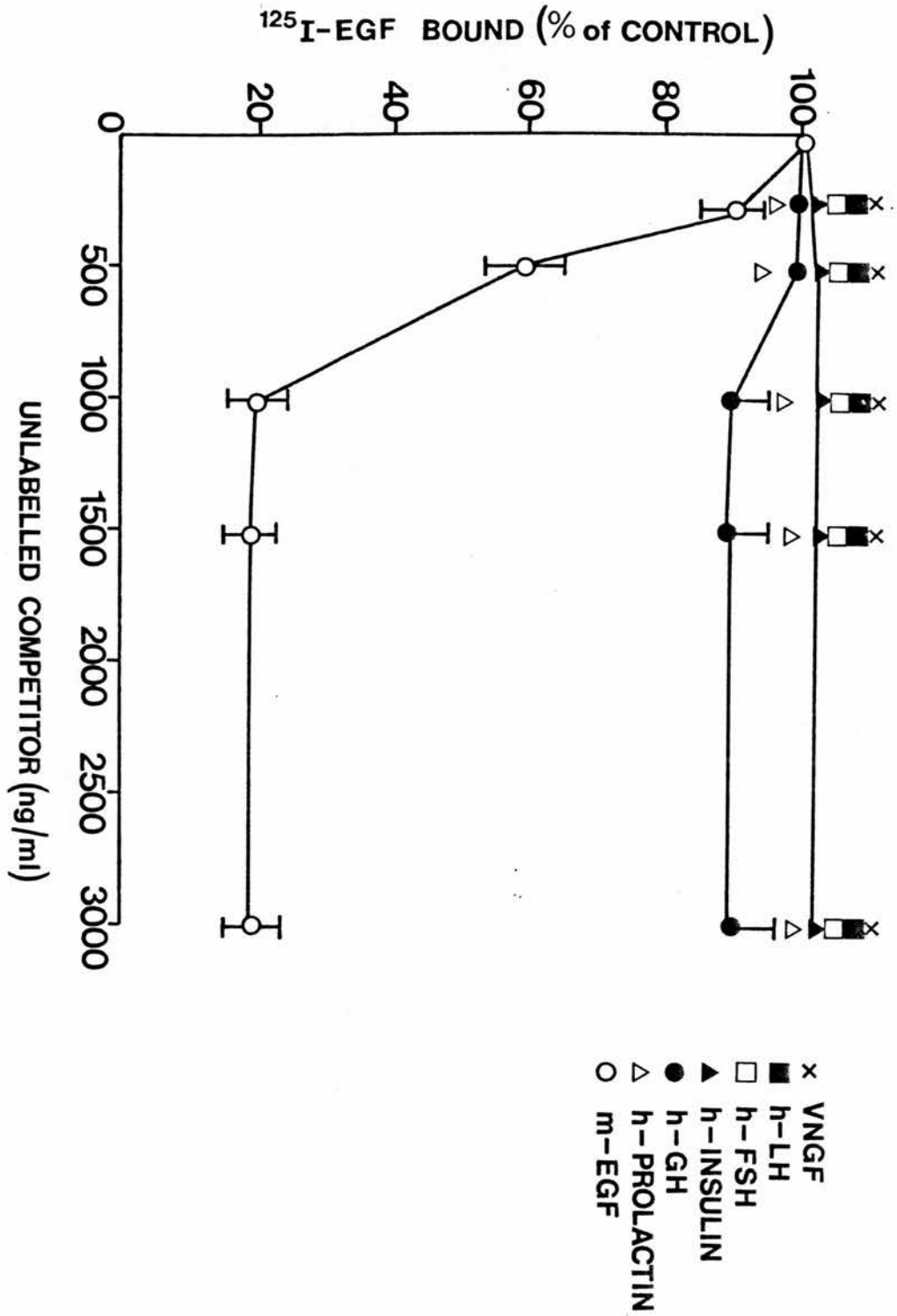

$^{125}$I-EGF BOUND (% of CONTROL)
100
80
60
40
20
0
0
500
1000
1500
2000
2500
3000
UNLABELLED COMPETITOR (ng/ml)
× VNGF
■ h-LH
□ h-FSH
▶ h-INSULIN
● h-GH
▷ h-PROLACTIN
○ m-EGF

Figure 19.

human PRL, GH, FSH, LH and venom nerve growth factor at concentrations between 250 - 3000 ng/ml were ineffective in competing with $^{125}$I-EGF for the particulate fraction receptors. This demonstrates the specificity of the EGF binding to its receptor in the BPH particulate fraction. However, human growth hormone was able to exhibit only a slight competition (<20%).

h) The impact of pH of incubation media on $^{125}$I-EGF binding to particulate fraction of BPH:

In order to find the optimal pH for $^{125}$I-EGF BPH binding, incubation media with various pH values were investigated, for their effect on EGF binding. Figure 20 shows that specific binding increased with increase in pH values, within the pH range of 2 - 7.4. Optimal pH observed for $^{125}$I-EGF binding to particulate fraction of BPH was 7.4. Level of binding dropped sharply at pH 8.0. At pH 9 binding had returned to basal level. These observations indicated that the influence of pH on EGF binding in the human prostate was considerable. Strict adherence to optimal pH is therefore important.

i) $^{125}$I-EGF binding: Thermal sensitivity of the EGF receptor protein:

The response of cells to heat shock has been known since the work of Ritossa (1962) on Drosophila embryo. Since then, studies on heat shock protein (HSP) have been carried out by other workers (Kelly and Schlesinger, 1978). Recently Hock and Hollenberg (1980); Ramani et al (1986) looked at the thermal sensitivity of the EGF receptor in the human placenta at higher temperatures. They observed an irreversible inactivation of the receptor at 65$^{o}$C.

These studies prompted an investigation to find out the effect of higher temperatures on EGF receptor binding. Figure 21 shows that when the particulate fraction was preheated at 45$^{o}$C for 10 minutes

Figure 20.

The impact of pH of incubation media on $^{125}$I-EGF binding to particulate fraction of BPH

The method is described under section 2.12(h).

Briefly, BPH particulate fractions (1 mg/ml protein) were incubated with 8.0 nmol/L for 90 minutes at 37°C in the presence and absence of 50-fold excess unlabelled EGF in media of various pH values. 10mM Tris-HCl pH 11 was adjusted to give pH values of 2 - 11 for use.

The particulate fraction incubated in medium pH of 7.4 served as control. Specific binding for each pH was calculated as described under "Materials and Methods" (section 2.12(h)).

Values are means of 3 different specimens, each analysed in duplicate but expressed as % of control value.

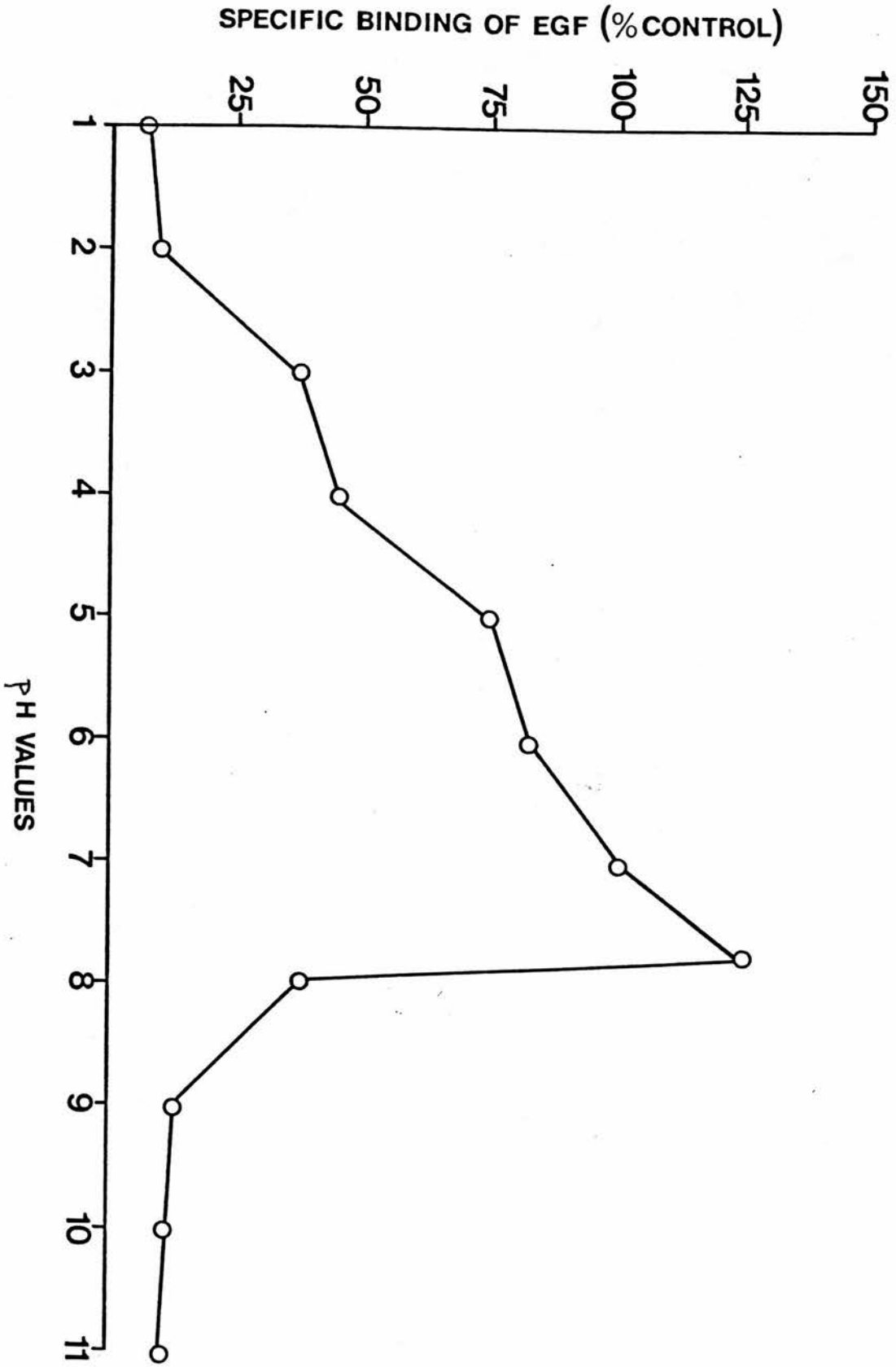

SPECIFIC BINDING OF EGF (%CONTROL)
25
50
75
100
125
150
1
2
3
4
5
6
7
8
9
10
11
PH VALUES

Figure 20.

Figure 21.

Thermal sensitivity

Prior to receptor binding assay, particulate fractions were preincubated at 45°C, 65°C, 75°C and 95°C for 10 minutes. Another fraction was preincubated at 37°C and that served as control. Thereafter, aliquots of the treated samples were incubated with $^{125}$I-EGF as detailed under section 2.12(i).

Briefly, 100 µl of each treated sample, and 100 µl of 8.0 nmol/L $^{125}$I-EGF were incubated in the presence and absence of 50-fold excess unlabelled EGF at 37°C for 90 minutes. At the end of the incubation period, $^{125}$I-EGF bound complex was separated from free by PEG precipitation and centrifugation.

Specific binding was calculated by the difference between non-specific and total binding.

Values are the means of 3 different samples, each analysed in duplicate.

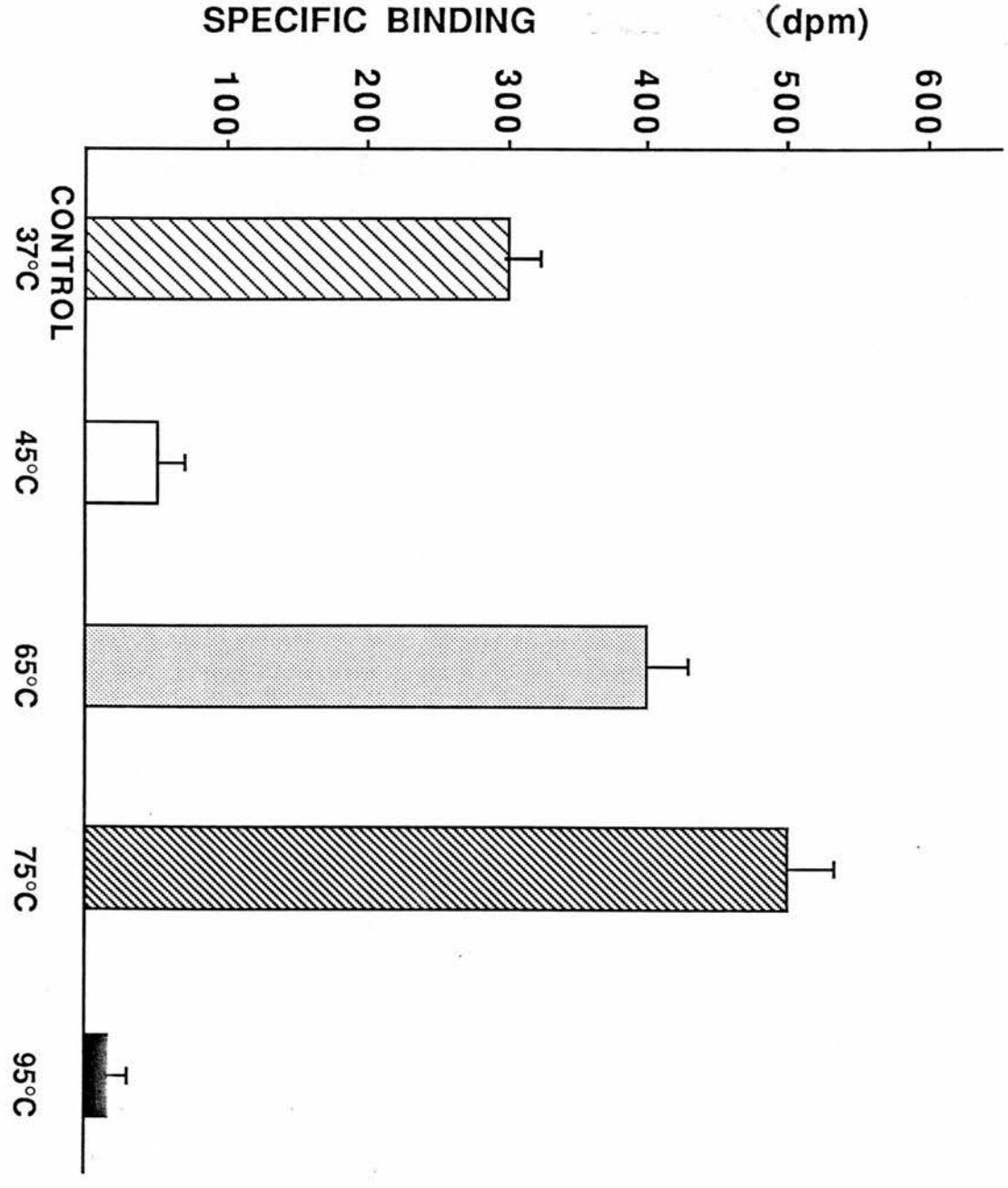

SPECIFIC BINDING
(dpm)
100
200
300
400
500
600
CONTROL
37°C
45°C
65°C
75°C
95°C

Figure 21.

prior to binding studies, the level of binding fell by up to 80% of control value. But heating the same particulate fraction at 65°C, and 75°C for 10 minutes prior to binding studies, it was observed that the level of binding unexpectedly rose again quite significantly by 33% and 66% respectively of control value. Total inactivation of the receptor was not realised until the particulate fraction was preheated at 95°C for 10 minutes prior to binding studies.

In another experiment, incubation of the particulate fraction and $^{125}$I-EGF mixture took place at 37°C, 65°C, 75°C and 95°C for 60 minutes, using 37°C as control. Figure 22 shows that incubation at 37°C revealed a steady rise in binding and formed a plateau after 45 minutes incubation which was maintained to 60 minutes, but at 75°C binding rose sharply by 40% of control level. But this rise was found to be only transient because it fell sharply after 20 minutes by 60% of control level. The same picture was observed at 65°C but the rise at 10 minutes was slightly lower and the fall slower. However, after 30 minutes the fall had reached almost the same level as that of the 75°C. Gradual fall in binding continued in both temperatures to 60 minutes. Because there was complete inactivation of receptors at 95°C, no binding was observed at all at that temperature.

j) Effect of enzyme and enzyme inhibitors on EGF binding with BPH:

It is well documented that the human prostate contains high levels of endogenous proteases (Gotterer et al, 1956; Mann and Mann, 1981; Isaacs and Coffey, 1984). It was therefore decided to examine the effects of some proteolytic enzymes and enzyme inhibitors on EGF binding with BPH.

Figure 22.

$^{125}$I-EGF binding: Effect of thermal EGF receptor sensitivity

The procedure is described under section 2.12(i).

Briefly, 100 µl aliquots of $^{125}$I-EGF and 100 µl aliquots of BPH particulate fractions were placed in various incubation tubes and incubated at 65°C, 75°C and 95°C from 0 - 60 minutes, but control sample was incubated at 37°C. At 10-minutes intervals, specific binding was assessed for the various temperatures after PEG precipitation and centrifugation and counting.

Values are means SD of 6 different samples, each analysed in duplicate but expressed as % of control.

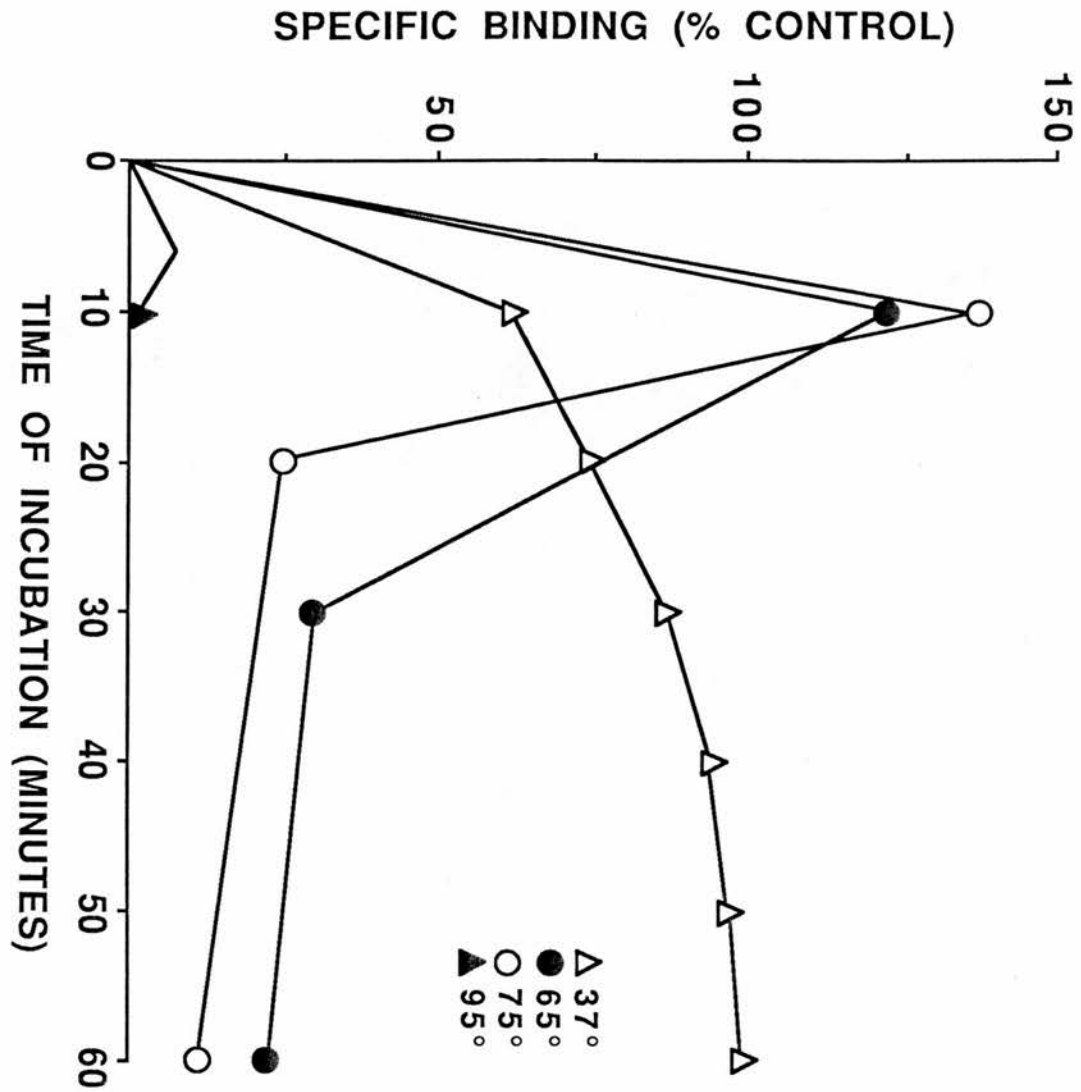

SPECIFIC BINDING (% CONTROL)
0
50
100
150
TIME OF INCUBATION (MINUTES)
0
10
20
30
40
50
60
37°
65°
75°
95°

Figure 22.

Figure 23 shows that pretreatment of particulate fraction of BPH with trypsin and $\alpha$ chymotrypsin completely digested the receptor protein resulting in total loss of binding. However, simultaneous addition of soybean antitrypsin completely reversed the trypsin induced binding losses and improved the binding by 10% over control values. Leupeptin and phenylmethylsulphonylfluoride (PMSF) enhanced the binding by 15% and 10% respectively over control values whilst DNASE and aprotinin had no effect on the binding.

These reports indicate that leupeptin, PMSF and soybean are effective enzyme inhibitors of the human prostate proteases.

k) $^{125}$I-EGF binding: Effect of BPH particulate fraction pretreatment with $MgCl_2$ and dextran coated charcoal (DCC):

$MgCl_2$ is known to dissociate tightly bound endogenous ligand receptor complexes and DCC to remove possible binding inhibitors from tissue extracts (Kelly et al, 1979). Consequently, Leake et al (1983) used these reagents to improve prolactin binding in the human prostate, suggesting the presence of endogenous prolactin in the human prostate. However, Traish et al (1987) did not observe any improvement in EGF binding in the rat prostate, also suggesting absence of endogenous EGF in the rat prostate.

In view of the fact that $MgCl_2$ and DCC reagents have not been used yet to test for the presence of endogenous EGF in the human prostate, it was decided to use the reagents to investigate if the human prostate contained any endogenous EGF.

Figure 24 shows that $MgCl_2$ pretreatment resulted in increases of both total and non-specific binding (non-specific binding being more than total), whereas DCC pretreatment inhibited binding with the overall impact being that of net loss in specific binding. In the case of BPH (Figure 24A) $MgCl_2$ pretreatment produced 70% fall

Figure 23.

$^{125}$I-EGF binding: Effect of pretreatment of BPH particulate fraction with enzymes or enzyme inhibitors

The method for this investigation is described in section 2.12(j).

Briefly, BPH particulate fractions were pretreated with enzymes or enzyme inhibitors, at 37°C for 60 minutes. After washing, the treated samples were tested for $^{125}$I-EGF binding at 37°C for 90 minutes, using 8.0 nmol/L $^{125}$I-EGF in the presence and absence of 50-fold excess unlabelled EGF. The control fraction was preincubated in buffer B only but was subjected to the same steps as the test fractions.

The following are the enzyme or enzyme inhibitors and the corresponding quantities used in this experiment. Quantities are expressed per mg protein:-

1 mg trypsin and DNASE, 2 mg soybean trypsin inhibitor, 1 mg phenylmethylsulphonylfluoride, 0.1 mg aprotinin, 0.05 leupeptin, 1 mg $\alpha$ chymotrypsin, all per mg of protein.

Details of the procedure are described under section 2.12(j).

Values are expressed at % of control value, but are means of 3 different samples, each analysed in duplicate.

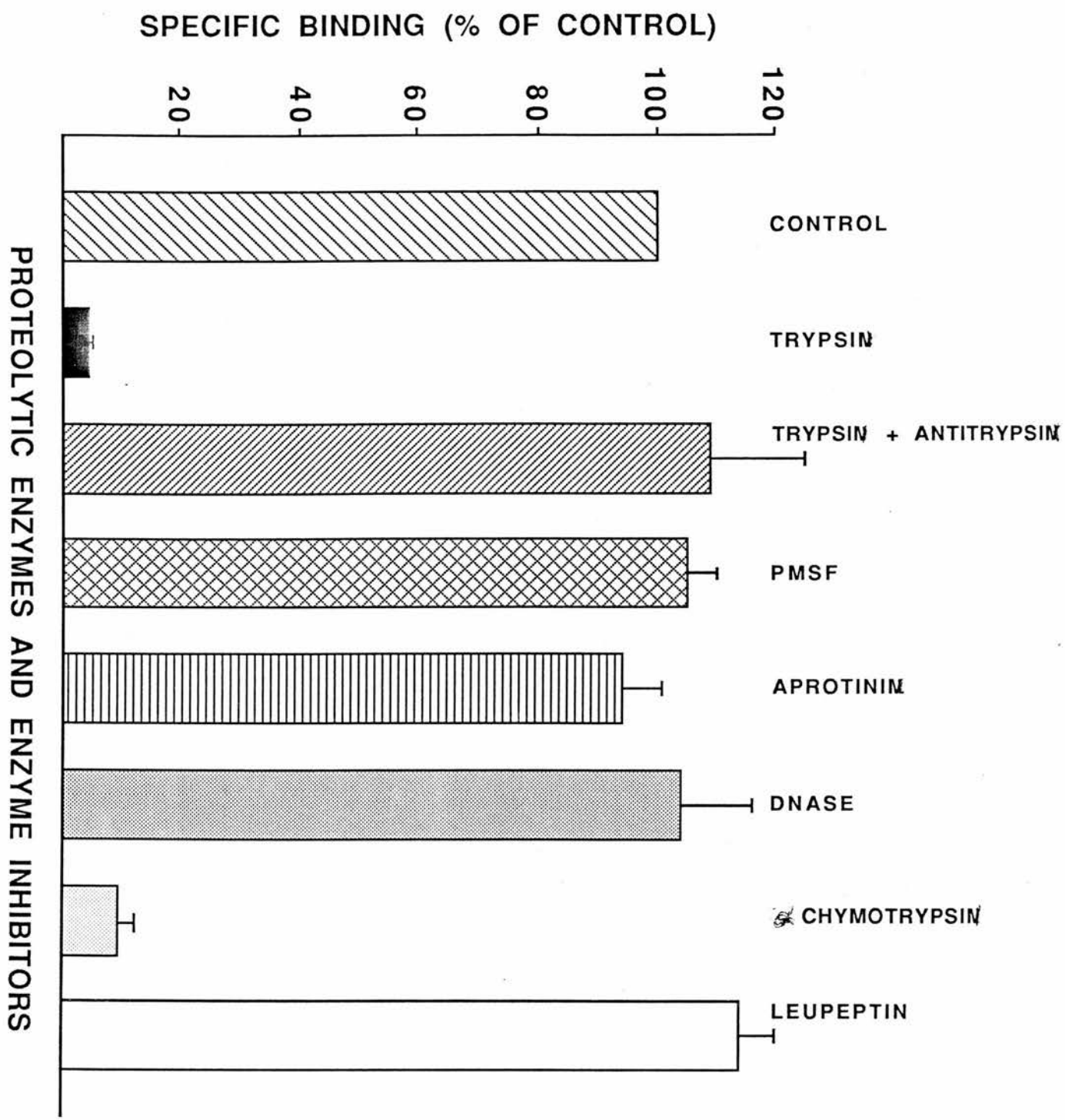

SPECIFIC BINDING (% OF CONTROL)
20
40
60
80
100
120
PROTEOLYTIC ENZYMES AND ENZYME INHIBITORS
CONTROL
TRYPSIN
TRYPSIN + ANTITRYPSIN
PMSF
APROTININ
DNASE
CHYMOTRYPSIN
LEUPEPTIN

Figure 23.

Figure 24.

$^{125}$I-EGF binding: Effect of $MgCl_2$ and DCC pretreatment of particulate fraction:

Two tissues were used: (A) human prostate, (B) human placenta.

The procedure for this experiment has been described under section 2.12(k).

Briefly, after BPH particulate fraction (Figure 24A) pretreatment with $MgCl_2$ or DCC, 100 µl aliquots of the treated samples were subjected to $^{125}$I-EGF receptor binding assay as described under section 2.12(k).

The experiment was repeated using human placenta particulate fraction (Figure 24B). Specific binding obtained on BPH control sample and the treated samples were compared (Figure 24A). Results obtained on the human placenta were also compared (Figure 24B).

Values are means of 4 different samples, each analysed in duplicate.

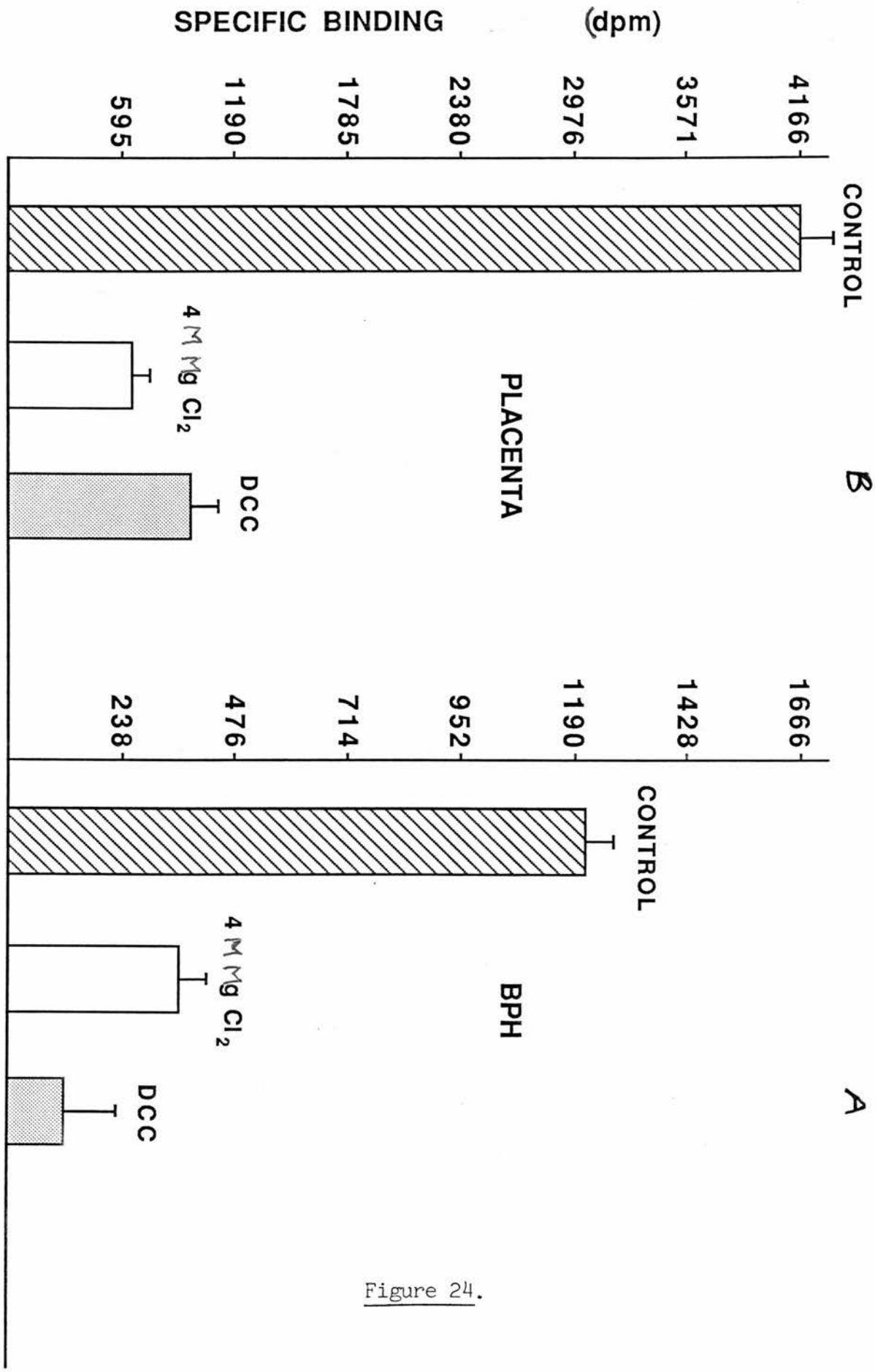

SPECIFIC BINDING (dpm)
4166
3571
2976
2380
1785
1190
595
CONTROL
4 M Mg Cl2
DCC
PLACENTA
B
1666
1428
1190
952
714
476
238
CONTROL
4 M Mg Cl2
DCC
BPH
A

Figure 24.

whilst DCC produced 90% in specific binding respectively. Placenta (Figure 24B) on the other hand exhibited about 84% fall after $MgCl_2$ pretreatment and 70% fall after DCC pretreatment, all compared with control values. The use of these reagents therefore indicated that the human prostate does not contain endogenous EGF.

1) $^{125}$I-EGF binding: Effect of storage at -70°C on prostate tissue and particulate fraction:

The human prostate is known to contain a lot of proteolytic enzymes (Gotterer et al, 1956; Mann and Mann, 1981; Isaacs and Coffey, 1984). It is also known that the enzymes are activated during storage and therefore storage may play a role in the physical and proteolytic degradation of receptors in the prostate tissue. In the course of scientific work, it is inevitable that tissues or particulate fraction are kept for later use. In fact this is the practice of many workers. In view of all these considerations, it was decided to look at the effect of storage at -70°C on prostate tissues and particulate fraction for 16 weeks. Figure 25 shows that at the end of 16 weeks, specific binding of both tissue and particulate fraction were still within reasonable range of the starting point. In the first few weeks of storage, however, binding rose above the starting level. After the second and fourth weeks, binding by tissue and particulate fraction respectively started to settle by approaching the starting level. The initial increase was due to alterations in non-specific binding, whilst the total binding remained constant. This showed that storage at -70°C for 16 weeks did not affect the EGF specific binding with prostate tissue or particulate fraction.

Figure 25.

$^{125}$I-EGF binding: Effect of storage at -70°C on BPH tissue and particulate fraction:

The method of tissue preparation and particulate fraction preparation and $^{125}$I-EGF receptor assay procedures have been described under section 2.12(m)

Briefly, BPH tissue was divided into 2 halves. One half was cut into very small pieces and the other half homogenised as described under section 2.12(m) making it a particulate fraction prepared. Protein was determined in the particulate fraction and the level of protein adjusted to 1 mg/ml and divided up into Eppendorf tubes (0.5 ml aliquots).

100 µl aliquot of the particulate fraction was incubated with 100 µl aliquot of $^{125}$I-EGF in the presence and absence of 50-fold excess unlabelled EGF at 37°C for 90 minutes. Specific binding observed was noted as 100% control. Thereafter, the portion that was chopped into little bits, together with the particulate fraction in 0.5 ml aliquots (in Eppendorf tubes) were stored at -70°C. $^{125}$I-EGF receptor assay was performed on both tissue and particulate fractions at regular intervals for 16 weeks.

The experiment was performed on 3 different tissues and mean values have been expressed as % control.

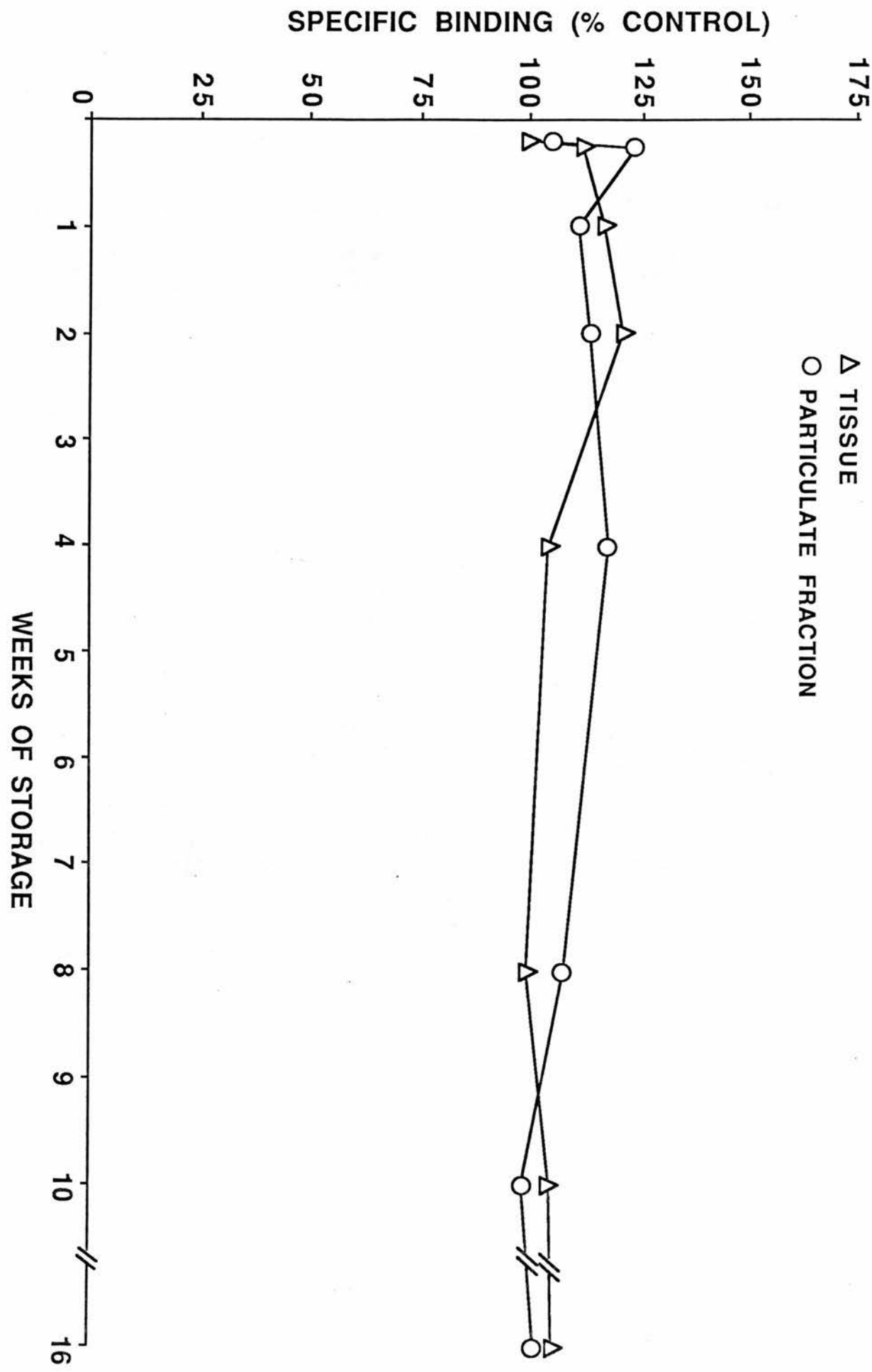

SPECIFIC BINDING (% CONTROL)
0
25
50
75
100
125
150
175
WEEKS OF STORAGE
1
2
3
4
5
6
7
8
9
10
16
Δ TISSUE
○ PARTICULATE FRACTION

Figure 25.

m) Binding of BPH particulate fraction to urogastrone and EGF:

Mouse EGF and human urogastrone have already been shown to be similar ligands (Savage, et al 1972; Gregory, 1975). This provided justification for using mEGF to interact with human BPH tissue, but interaction of urogastrone with human BPH failed to produce positive binding (Gregory et al, 1986). This prompted the author to investigate the binding characteristics between mEGF and human urogastrone with human BPH. The outcome of the investigations are now presented:-

TABLE 4 shows that both EGF and urogastrone showed specific binding with BPH particulate fraction, indicating that the two ligands recognised the same receptor and shared the same receptor sites. The use of urogastrone was therefore not the explanation for the absence of EGF receptors in the human BPH observed by Gregory et al (1986). However, as indicated in TABLE 4, urogastrone, whether in its labelled form or unlabelled form, when used for displacement always produced lower results than mEGF at any time. For example, BPH binding with $^{125}$ImEGF in the presence and absence of unlabelled mEGF produced specific binding of 3594 dpm, whereas only 815 dpm specific binding was achieved when the same reaction took place in the presence and absence of unlabelled urogastrone, thus underestimating the level of receptors by 77%. Furthermore, in another experiment BPH binding with $^{125}$I-urogastrone in the presence and absence of unlabelled mEGF produced 2078 dpm specific binding, whereas only 775 dpm specific binding was obtained in the presence and absence of unlabelled urogastrone, here again underestimating the receptor level in the prostate by 63%. These results mean that even though urogastrone and mEGF recognise and bind the

TABLE 4.

BINDING OF BPH PARTICULATE FRACTION TO $^{125}$I-mEGF AND $^{125}$I-UROGASTRONE

The procedure is described under section 2.12(m).

Briefly, 100 µl $^{125}$I-EGF was incubated with 100 µl BPH particulate fraction (1 mg/ml protein) in the presence and absence of 50-fold excess unlabelled EGF or unlabelled urogastrone at 37°C for 90 minutes (Column A).

In another experiment 100 µl $^{125}$I-Urogastrone was incubated with 100 µl BPH particulate fraction in the presence and absence of 50-fold excess unlabelled m EGF or urogastrone at 37°C for 90 minutes (Column B).

At the end of the incubation period $^{125}$I-ligand bound complex was separated from free by PEG precipitation and centrifugation. Specific binding was calculated by the difference between non-specific and total bindings.

Results were reproduced on 3 different samples and expressed as means ± SD.

TABLE 4.

| | $^{125}$I LIGAND | UNLABELLED mEGF | UNLABELLED UROGASTRONE |
|---|---|---|---|
| A | mEGF | SPECIFIC BINDING 3594 dpm ± 200 | SPECIFIC BINDING 815 dpm ± 70 |
| B | UROGASTRONE | 2078 dpm ± 100 | 775 dpm ± 50 |

same receptor, urogastrone underestimates the EGF receptors in the human BPH by as much as 70%. This, therefore, may provide some explanation for the negative results obtained by some workers. The reason for the different binding characteristics may be due to differences in the affinities of the two ligands for the EGF receptor in the human BPH tissue.

3.4 Molecular characterisation of the EGF receptor:

Two techniques were employed:-

a) Affinity labelling and chemical crosslinking of EGF to its receptor

b) Phosphorylation of the EGF receptor

a) Affinity labelling and crosslinking of $^{125}$I-EGF receptor, SDS - PAGE and autoradiography:

The specific high affinity receptor is a single chain polypeptide with a molecular weight in the range 150,000 - 170,000 daltons. To determine whether the prostatic EGF receptor has a similar molecular weight, the experiments detailed under section 2.13 were carried out. Figure 26 (Lane A) shows that $^{125}$I-EGF was covalently attached to proteins with approximate molecular weights in the range 150,000 and 170,000 daltons, which was identical to the molecular weight of the classical molecular weight of the EGF receptor. These complexes appear to be specific for EGF since labelling in the presence of excess unlabelled EGF resulted in disappearance of the bands (Figure 26; Lane B). Specific labelling of some low molecular weight proteins were also seen. The relevance of these is not immediately clear; but they could represent the cytoplasmic domain of EGF receptors since they occupy the same position as albumin as demonstrated by molecular weight markers. This finding could be confirmed using the newly developed monoclonal antibody to the internal domain (F4) in immunoprecipitation studies.

b) Stimulation of membrane protein phosphorylation by EGF:

Crosslinking of the $^{125}$I-EGF receptor revealed that $^{125}$I-EGF was bound specifically to both 170,000 and 150,000 molecular weight species. Given these results it was next sought to determine whether the EGF binding would also stimulate the phosphorylation of the observed 170,000 and 150,000 molecular weight species.

Figure 26

Affinity labelling: Crosslinking of $^{125}$I-EGF, SDS - PAGE and autoradiography

The methods for these investigations have been described under section 2.13, but briefly, 100 µl aliquots of plasma membrane were incubated with 100 µl aliquots of $^{125}$I-EGF (8.0 n mol/L) in the absence (Lane A) or presence (Lane B) of 50-fold excess unlabelled EGF in 200 µl HEPES buffer at 37$^{o}$C for 90 mins. At the end of the incubation period, 1 ml HEPES buffer was added to the medium to stop the reaction. After centrifugation to separate $^{125}$I-EGF bound complex from free, the labelled bound complex was crosslinked using the chemical crosslinker DSS prior to SDS - PAGE followed by autoradiography, using intensifying screen and x-ray hyperfilm as described under section 2.13.

Similar results were obtained on 3 different samples.


Figure 26.

Figure 27 shows the result of the phosphorylation experiment which was performed in the presence of EGF ( Lane A ) and absence of EGF ( Lane B ). Since a band has appeared in the 170,000 molecular weight range in Lane A but not in Lane B, it only follows that EGF has stimulated phosphorylation of the EGF receptor. It also follows that in vitro phosphorylation does not take place in the human prostate in the absence of EGF.

3.5 Immunocytochemical techniques:

The development of monoclonal antibody to the external domain of the EGF receptor (Waterfield et al, 1982) has enabled many workers to measure specifically EGF receptors in a whole range of human and animal tissues and cells, employing the technique of immunocytochemistry, but no-one has used the technique to measure EGF receptors in the human BPH tissue, yet. It was therefore decided to confirm the presence of the EGF receptors in the human prostate tissue by immunocytochemistry involving the use of the monoclonal antibody to the EGF receptor sites (external domain). Two immunocytochemical techniques were used: a) the indirect immunoperoxidase method and b) the labelled avidin biotin technique. These techniques were used to demonstrate the presence of the EGF receptor in the prostate.

a) Plates 1 and 2 illustrate the pattern of immunoperoxidase reactivity observed in BPH. Plate 1 shows a negative control in which the use of the monoclonal antibody was omitted. Plate 2 shows positive staining in which the monoclonal antibody was used. Staining by the antibody was confined to the basal layers of the epithelial cells whilst the adjacent stroma remained clear. There was no apparent nuclear staining but the immunoperoxidase fraction was limited to cell membranes. Staining of the epithelial cells was

Figure 27

Stimulation of membrane protein phosphorylation by EGF

Details of the procedure are described under section 2.13(b).

Briefly, BPH membrane was first extracted for 30 minutes with Triton buffer (1% $^{v}/v$) pH 6.8, and the extract incubated in the presence ( Lane A ) and absence ( Lane B ) of 60 ng unlabelled EGF at room temperature.

At the end of the incubation period, phosphorylation was performed for 5 minutes in the presence of 10 µCi ($\gamma^{32}P$) ATP. The products were then analysed by SDS - PAGE followed by autoradiography.

The experiment was performed twice.

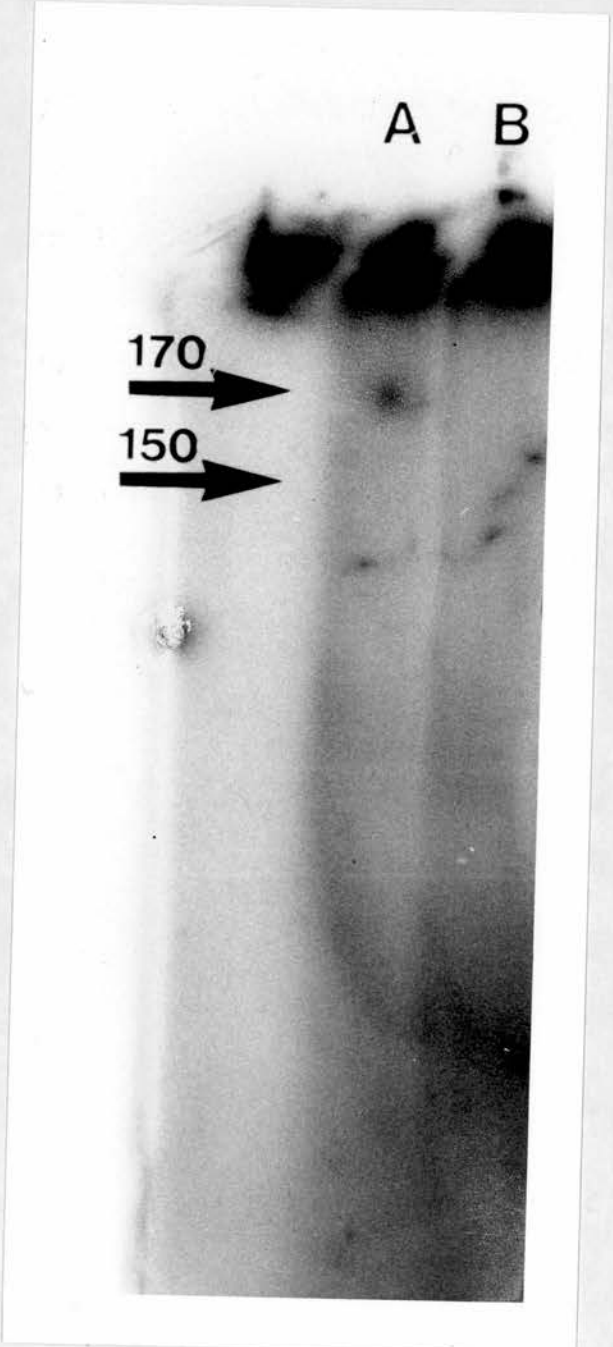



Figure 27.

Plates 1 and 2

Indirect immunoperoxidase method, using monoclonal antibody to the external domain of EGF receptor

Details of the technique are described under section 2.14.

Briefly, 3 - 4 um cryostat sections were fixed in acetone for 20 minutes. Sections were thereafter treated with primary antibody (1/30 dilution). This was followed by secondary antibody conjugated with peroxidase. Reaction was detected by diaminobenzidine (DAB) in the presence of hydrogen peroxide.

Sections were counterstained with dilute haematoxylin and finally mounted in DPX.

Plate 1 was not treated with the monoclonal antibody and therefore served as negative control.

Plate 2 was treated with the monoclonal antibody and therefore indicates positive staining.

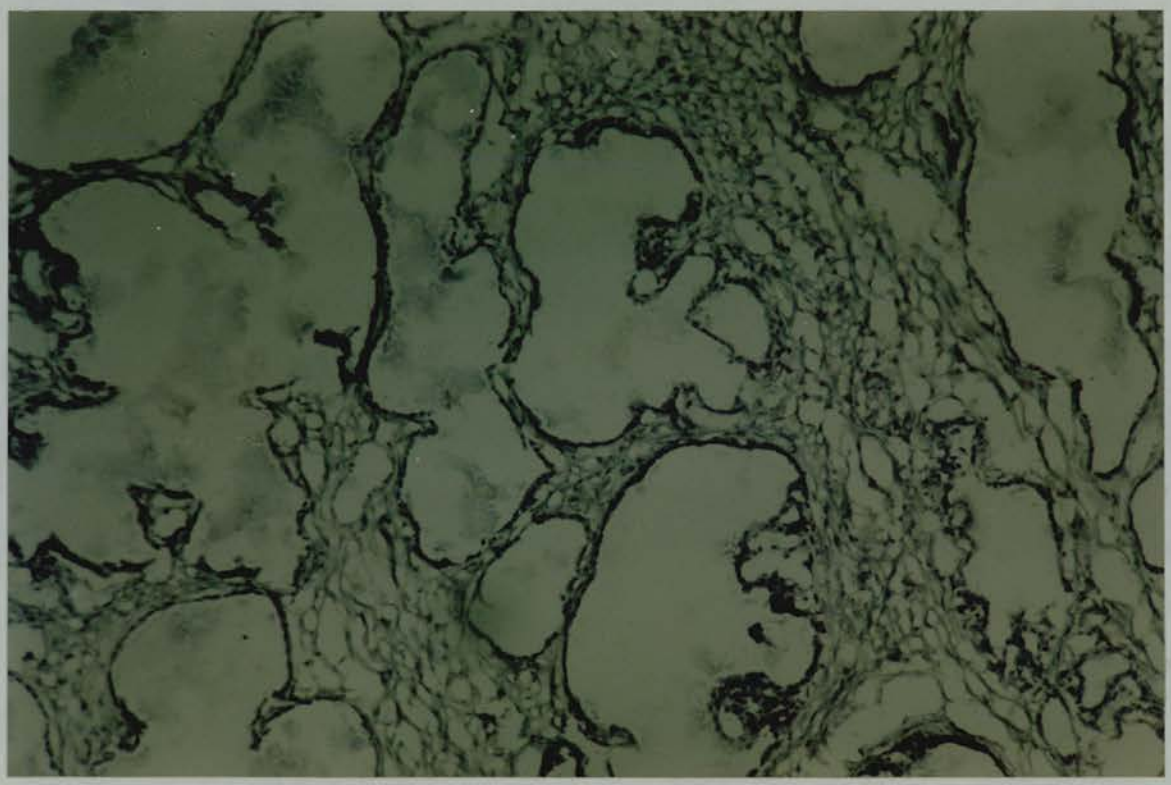


Plate 1A (x 133)

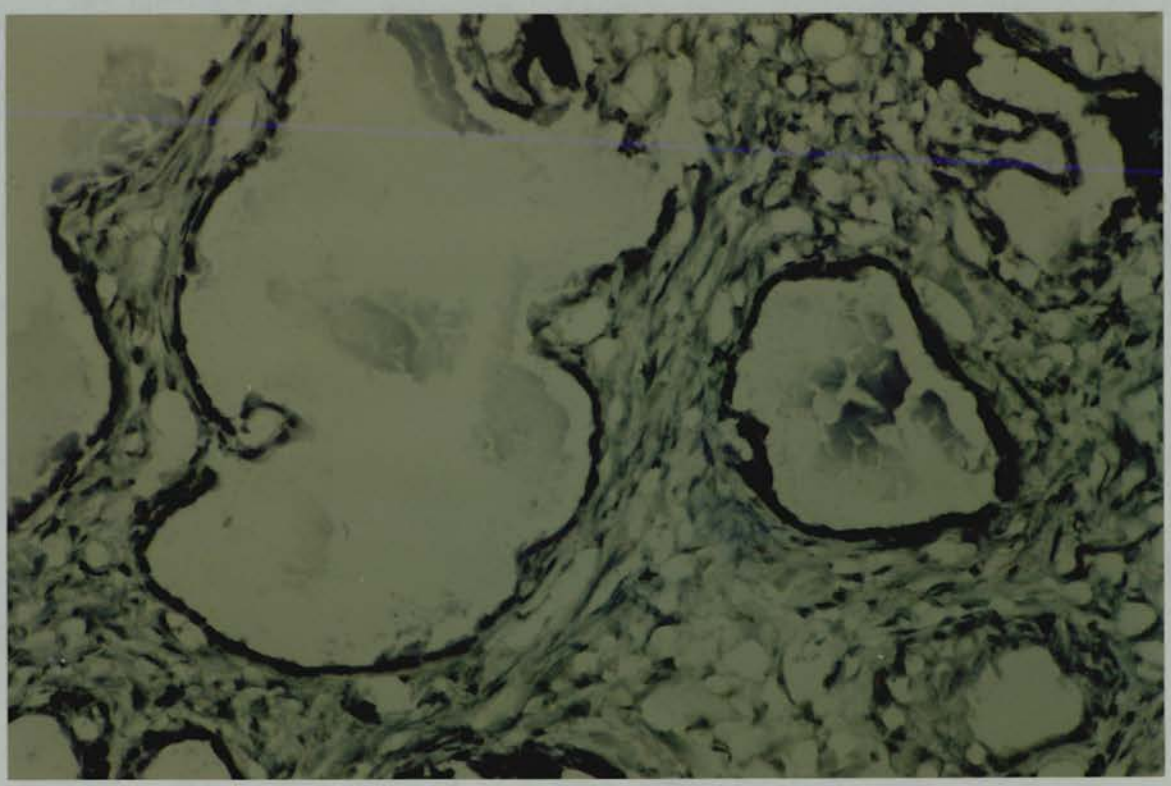


Plate 1B (x 333)

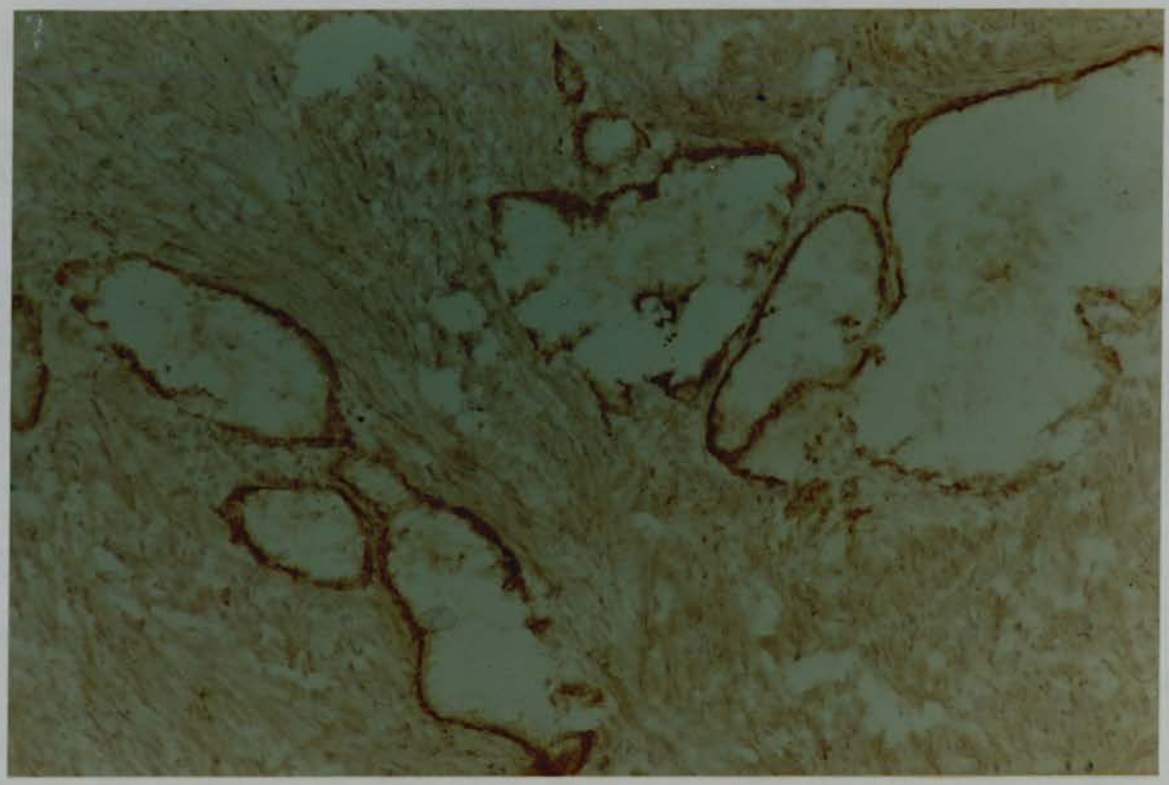


Plate 2A (x 133)

Plate 2B (x 333)

predominantly uniform throughout the same specimen, but there was considerable variability in the intensity of staining between different tissues. Staining of sections was assessed by three independent observers and the intensity was graded as negative (-), moderate (+) or intense (++) - (+++).

b) Labelled avidin biotin technique: Results were identical to those produced by the indirect immunoperoxidase technique. Plate 3 is showing a negative staining.

Positivity was indicated by a red colour and was confined to the basal layers of the epithelial cells. The stromal areas and the nuclei were stained slightly. Staining of sections was semiquantitatively scored as described above (Plate 4).

c) Comparison between immunocytochemistry and radioligand assay:

The results of the two immunocytochemical techniques were compared with those obtained using radioligand assay technique. Figure 28 shows that there was very good correlation between the intensity of staining and receptor positivity as measured by ligand exchange assay. Agreement between the two techniques occurred in all ten tissues examined. Six specimens demonstrated intense immunocytochemical staining (> 6 dpm/l x $10^{-6}$) whereas moderate or negative staining corresponded to binding less than (6 dpm/l x $10^{-6}$).

Plates 3 and 4

Labelled avidin-biotin method using MAB to the external domain of the EGF receptor

The method used has been outlined under section 2.14(b).

Briefly, 3 - 4 µm BPH frozen sections were fixed in acetone.

Sections were then treated with primary antibody (1/30 dilution) for 24 hours. Sections were next subjected to treatment with sheep antimouse biotinylated immunoglobulin and subsequently followed by streptavidin alkaline phosphatase.

Colour was developed by fast red ITR in the presence of fresh alkaline phosphatase substrate (naphthol ASBI phosphate).

Sections were counterstained and mounted in glycerine jelly.

Plate 3: Showing BPH stained section, negative control.

Plate 4 Showing BPH stained section, positive control.

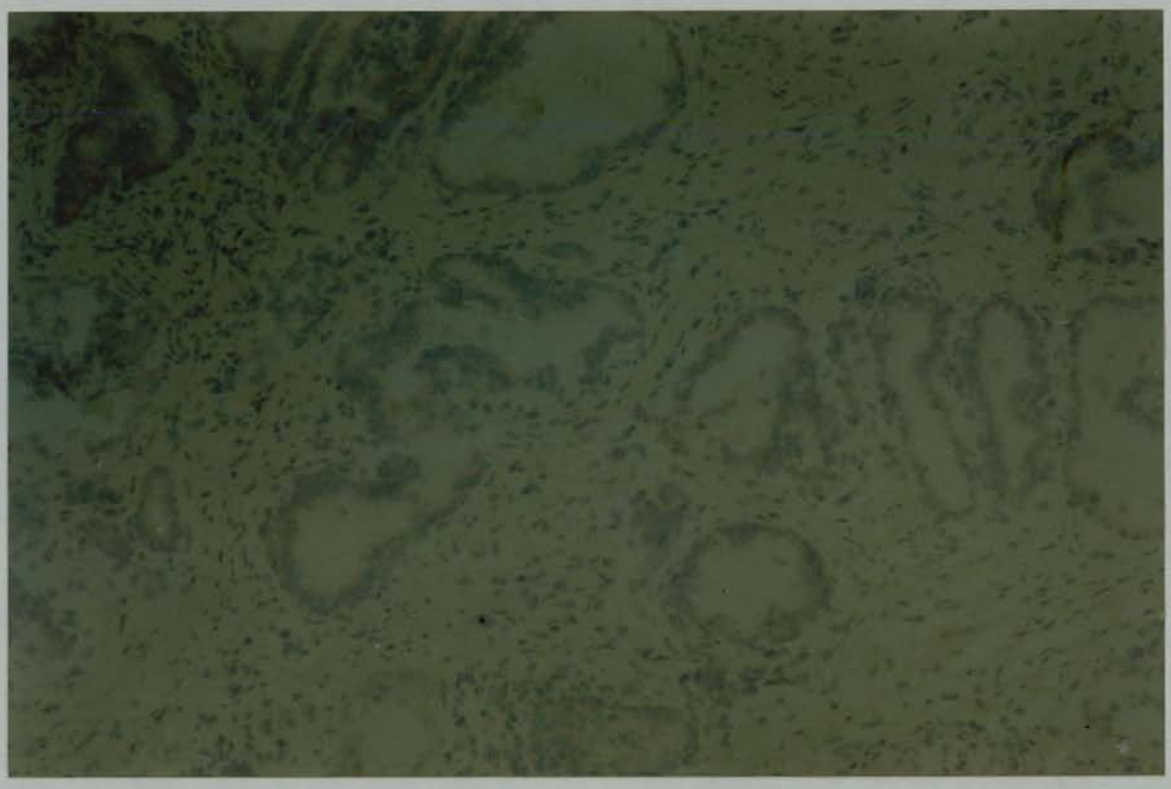


Plate 3A (x 133)

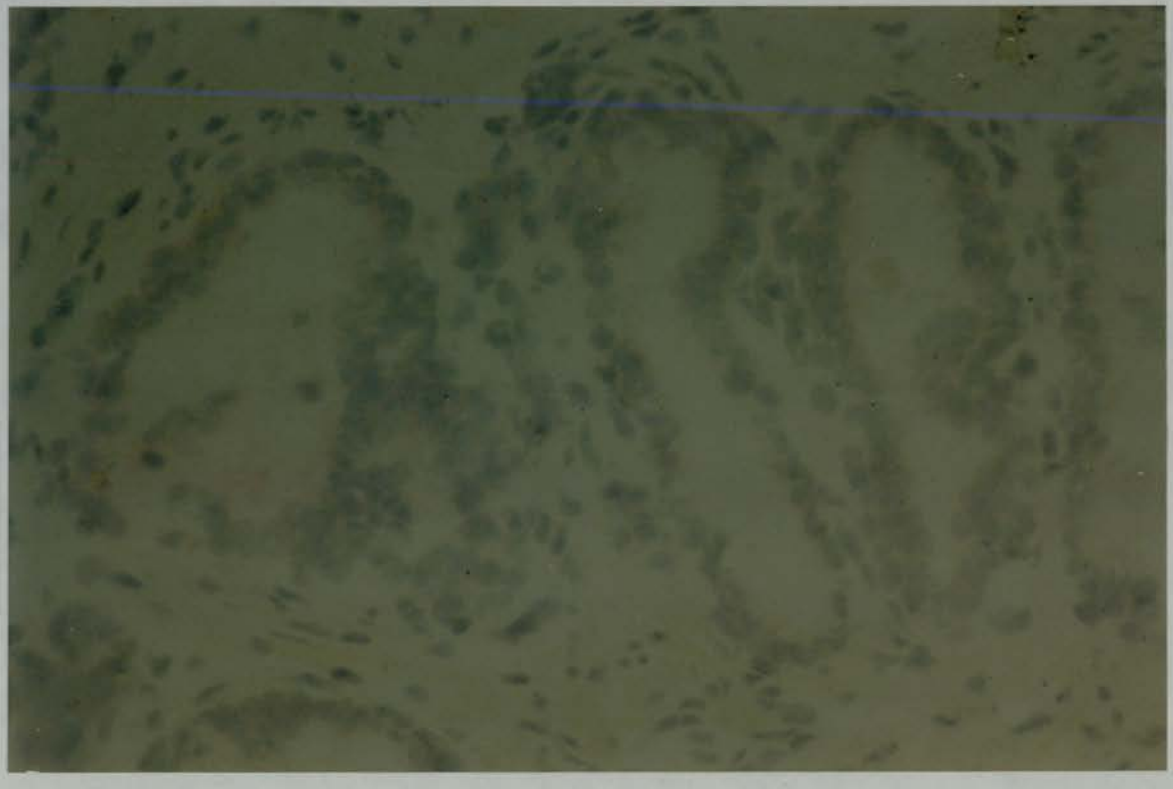


Plate 3B (x 333)

Plate 4A (x 133)

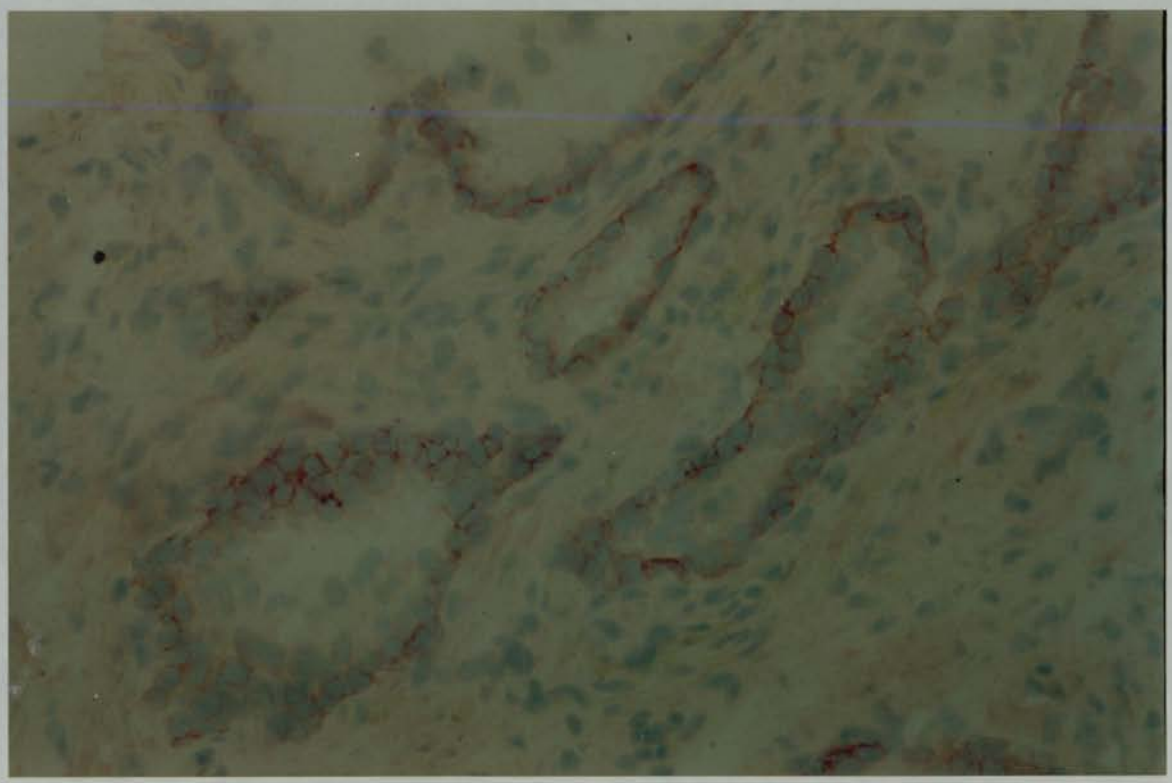


Plate 4B (x 333)

Figure 28

Comparison between immunocytochemistry and radioligand assay on BPH tissue

BPH tissues were analysed for EGF receptor levels by two techniques and these are (a) Immunocytochemistry and (b) Radioligand assay.

Details of the two techniques are described under section 2.11 (radioligand assay) and section 2.14(a)(immunocytochemistry).

Briefly (radioligand assay), $^{125}$I-EGF and BPH particulate fraction are incubated at 37$^{o}$C for 90 minutes in the presence and absence of unlabelled 50-fold excess unlabelled EGF. After free and bound $^{125}$I-EGF complex were separated, specific binding was calculated (X axis).

With regard to immunocytochemistry, cryostat sections were treated with primary and secondary monoclonal antibodies. After counterstaining, the sections were mounted.

Results of the two techniques are compared.

Sections were viewed by 3 independent observers. (Y axis)

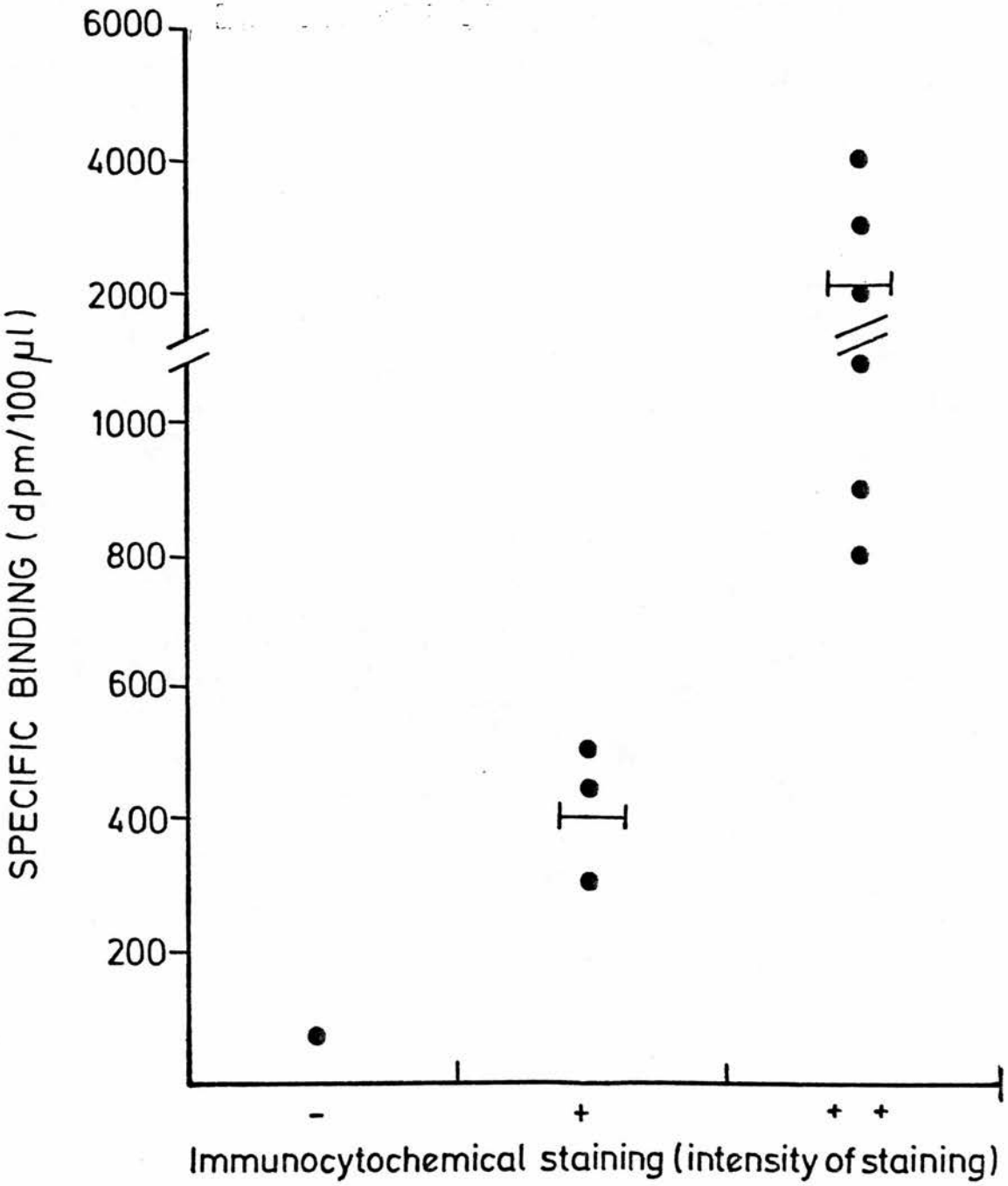

6000
4000
2000
1000
800
600
400
200
SPECIFIC BINDING (dpm/100µl)
-
+
+ +
Immunocytochemical staining (intensity of staining)

Figure 28.

3.6 Demonstration of the presence of EGF receptors in adenocarcinoma of the prostate:

Transformed cells show varying responses to exogenous EGF. This characteristic is influenced by the histologic grade of the tumour. Thus Bradley et al (1986) observed high EGF receptor levels in well-differentiated cells but low receptor levels were observed in poorly-differentiated cells. Sainsbury et al (1985) observed higher levels of EGF receptors in poorly differentiated breast tumour than he observed in other histologic grades.

This prompted the author to examine adenocarcinoma of the prostate for the presence of EGF receptors, using (a) radioligand assay and (b) immunocytochemical techniques: (i) Indirect immunoperoxidase and (ii) Labelled avidin biotin method. Monoclonal antibody to the external domain of the EGF receptor (EGF-$R_1$) was used in the immunocytochemistry.

Figure 29 shows that well differentiated CaP contains EGF receptors. The biochemical binding characteristics were found to be identical to those observed in BPH (section 3.3(f)) except that only one class of binding sites was observed in the CaP.

Plates 5 and 6 confirm the presence of the receptors in the well differentiated CaP. Plate 5 was stained by the immuno-peroxidase technique, whilst Plate 6 was stained by the labelled avidin technique.

Figure 29

Demonstration of EGF receptor in adenocarcinoma of the prostate

a) Radioligand assay technique

The methods were identical to those described under 2.12(e; f).

Briefly, 100 µl aliquots of CaP tissue particulate fraction were incubated with varying concentrations of $^{125}$I-EGF (0.5 - 12.0 nmol/L) in the presence and absence of 50-fold excess unlabelled EGF at 37$^{o}$C for 90 minutes.

At the end of the incubation period, specific binding was separated from non-specific by PEG precipitation and centrifugation as outlined under section 2.12(e). Concentration at which saturation occurred was noted (A).

Data obtained from the saturation studies detailed above was used in calculating the dissociation constant Kd by Scatchard plot analysis (Scatchard, 1949) (B).

Results are expressed as means of 4 different samples.

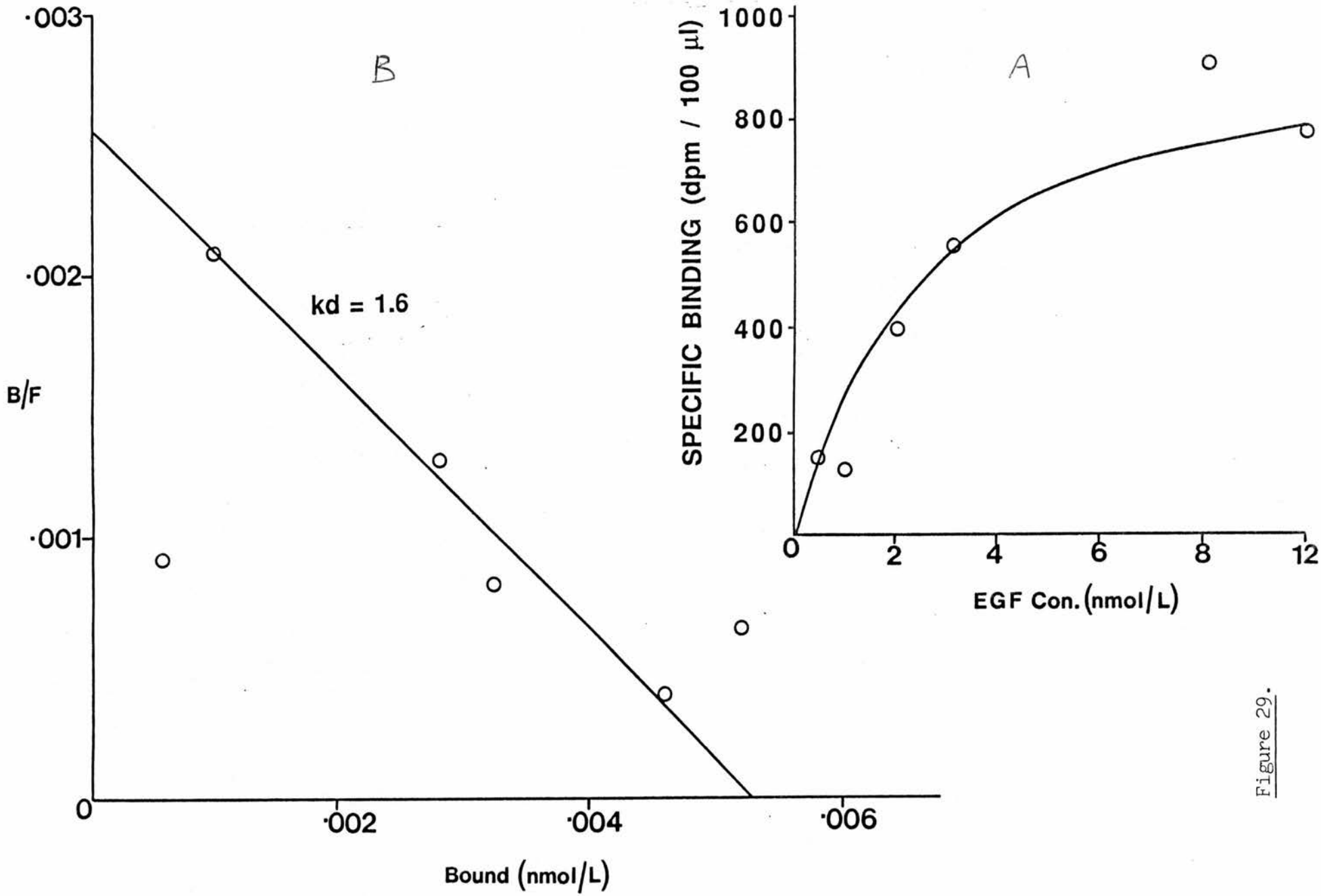

B
·003
·002
·001
0
B/F
kd = 1.6
·002
·004
·006
Bound (nmol/L)
A
1000
800
600
400
200
0
2
4
6
8
12
SPECIFIC BINDING (dpm / 100 µl)
EGF Con. (nmol/L)

Figure 29.

b) Plates 5 and 6

Demonstration of EGF receptors in CaP. Immunocytochemical method

i) Plate 5 Indirect immunoperoxidase technique

Details of the procedure are described under section 2.14(a).

Briefly, 3 - 4 µm cryostat sections were treated with monoclonal antibody to the external domain of the EGF receptor. This was followed by secondary antibody conjugated with peroxidase. Reaction was detected by diaminobenzidine (DAB) and finally counterstained with Harris haematoxylin and mounted in DPX (Plate 5).

ii) Plate 6 Labelled avidin biotin technique

Details of the method are outlined under section 2.14(b).

Briefly, 3 - 4 µm frozen sections were fixed in acetone. Sections were then treated with primary antibody (1/30 dilution) for 24 hours. After treatment with sheep antimouse biotinylated immunoglobulin followed by streptavidin alkaline phosphatase, colour was developed by fast red ITR in the presence of fresh alkaline phosphate substrate (napthol ASBI phosphate). Sections were counterstained and mounted in glycerine jelly (Plate 6).

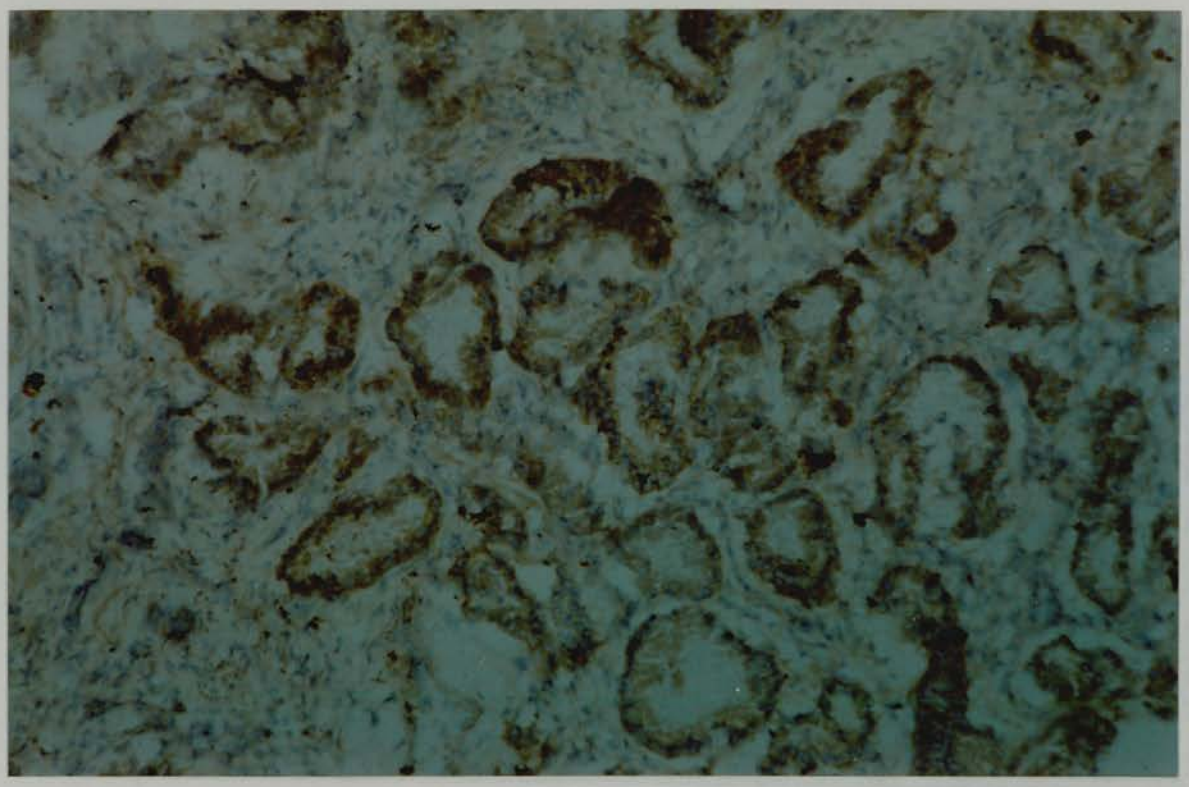


Plate 5A (x 133)

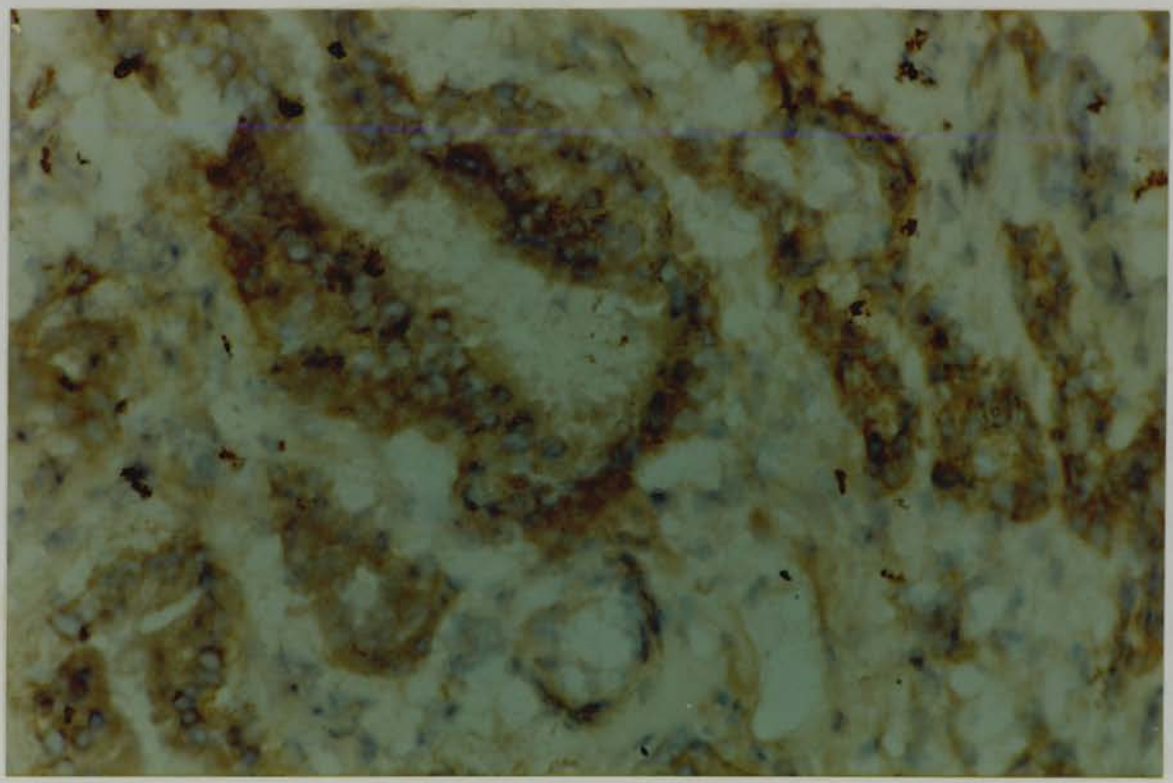


Plate 5B (x 333)

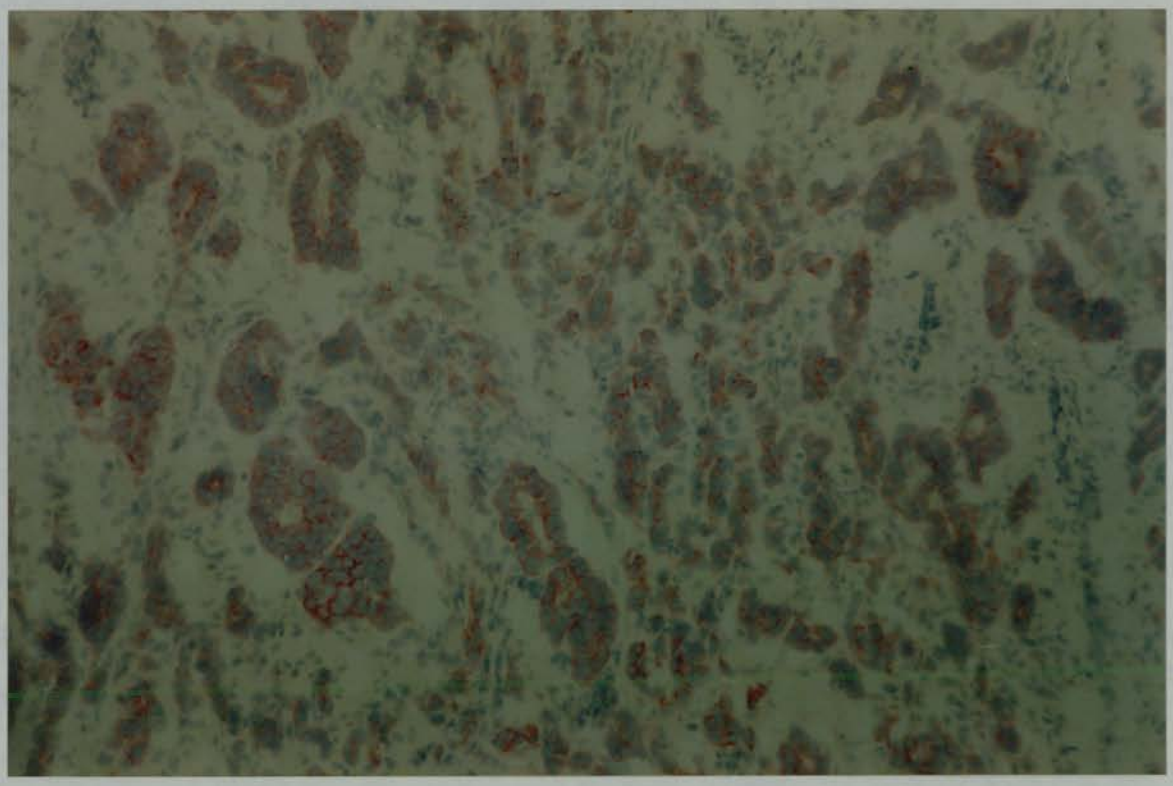


Plate 6A (x 133)

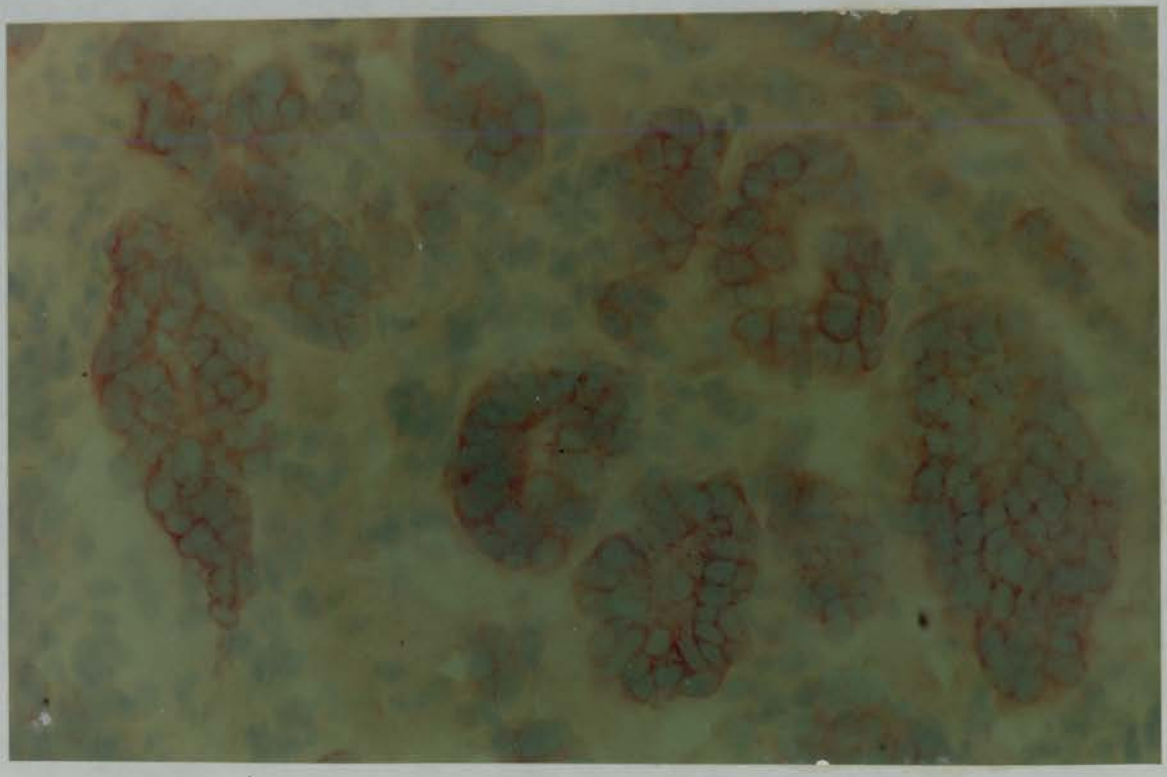


Plate 6B (x 333)

3.7 Comparison of EGF receptor levels in BPH and CaP:

Two techniques were used and these were:-

a) Radioligand assay technique

b) Immunocytochemical method -

i) Indirect immunoperoxidase method

ii) Labelled avidin biotin method

a) Radioligand assay:

Establishment of the presence of EGF receptors in both BPH and CaP prompted the decision to compare the receptor levels in BPH and CaP and also to correlate the EGF receptor levels with the various grades of the CaP.

18 BPH and 19 CaP tissues representing the various histological grades of CaP were selected for EGF receptor assay by radioligand assay technique. Figure 30A shows that the 18 BPH tissues contain an average of 125 fmol of $^{125}$I-EGF/mg protein. In contrast, the data on 19 patients with CaP shows a marked reduction in specific EGF binding (mean $\pm$ SD = 52 $\pm$ 11 fmol/mg protein) (Figure 30A). As demonstrated in Figure 30A, results of the two groups show a statistically significant difference between the mean levels of EGF receptor in BPH and CaP ($P<0.01$). The bulk of the BPH tissues (17/18) were found to be positive for EGF receptors, whilst only 10 out of 19 malignant tissues were found to be positive. But when the EGF receptor expression was correlated with the histologic grades of the malignant tissues, a strong relationship between the levels of EGF receptor binding and histological differentiation was observed (Figure 30B). Well-differentiated tumours (Gleason score 2 - 4) maintain

Figure 30

Presence of EGF receptors in BPH and CaP

a) Radioligand assay:

18 BPH and 19 CaP tissues were analysed by radioligand assay, as detailed under Section 2.11 for EGF receptors.

Briefly, 100 µl of 8.0 nmol/L $^{125}$I-EGF was incubated with 100 µl particulate fraction of either BPH or CaP at 37°C for 90 minutes. Separation of bound complex from free was achieved by PEG precipitation.
After counting radioactivity retained in the pellet, specific binding was calculated by subtracting non-specific from total binding.

Relationship between specific binding obtained in BPH and CaP was established. Also established was the correlation between specific binding and the histologic grades of the prostate tumour, based upon Gleason score.

A: Comparison of mean specific binding of BPH with CaP.

B: Comparison of mean specific binding with the histologic grades of CaP.

Values are means of 3 different samples, each analysed in duplicate.

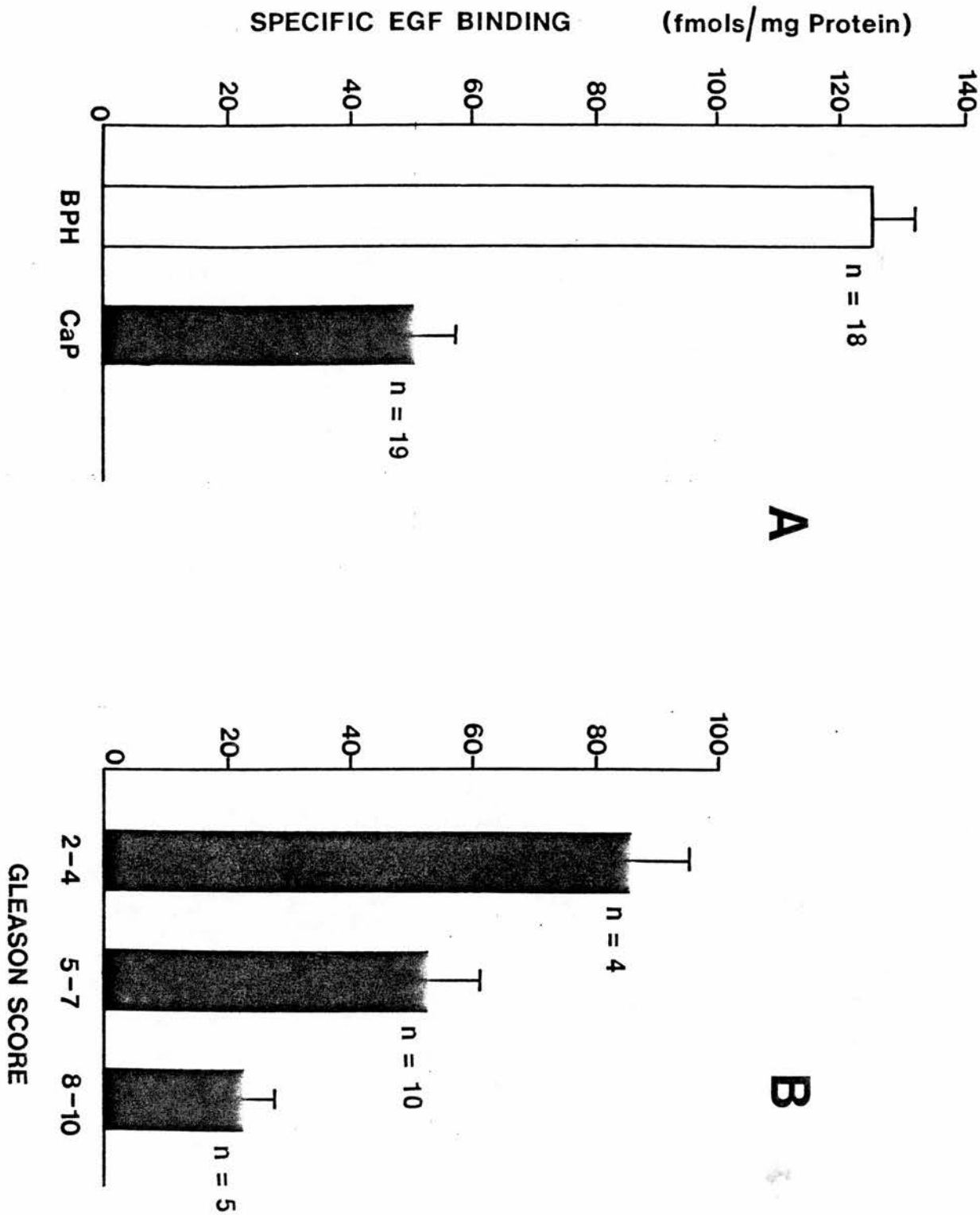

SPECIFIC EGF BINDING (fmols/mg Protein)
0
20
40
60
80
100
120
140
BPH
CaP
n = 18
n = 19
A
0
20
40
60
80
100
2-4
5-7
8-10
n = 4
n = 10
n = 5
GLEASON SCORE
B

Figure 30.

significantly larger concentrations of the receptor (mean ± SD = 84 ± 13 fmol/mg protein) than the poorly differentiated tumours (Gleason score 8 - 10) in which the majority of the tissues were devoid of EGF binding (Figure 30B).

b) Immunocytochemical methods:

i) Indirect immunoperoxidase technique using MAB EGF-$R_1$ (for external domain of EGF receptor):

Comparison of the EGF receptor levels in BPH and CaP was further confirmed by using indirect immunoperoxidase technique in which a monoclonal antibody specific to the binding site of the EGF receptor was used.

Plates 7 and 8 show that staining with the antibody was confined to the basal layers of the epithelial cells. Furthermore, BPH and well differentiated tumours (Gleason score 2 - 4) demonstrated an intense immunocytochemical staining (+++), whereas the poorly differentiated tumours (Gleason score 8 - 10) showed little or no staining at all (-) (Plate 9). Histologically the well differentiated CaP exhibited a marked increase in acinar glands which resulted in a decrease in interacinar stromal tissue compared with the large interacinar stromal spaces seen in BPH tissue. The intensity of staining was also observed to correlate very well with the degree of receptor positivity.

ii) Labelled avidin-biotin technique using MAB to the external domain (EGF-$R_1$) of EGF receptor:

The picture was identical to the one given by immunoperoxidase staining using DAB as chromogen described under section 3.7(b)(i). Briefly, positive staining of BPH indicated by red colour, was confined to the basal layers of the epithelial cells, whilst the adjacent stroma remained clear (Plate 10).

b) Plates 7, 8, 9: Presence of EGF receptors in BPH and CaP

i) Indirect immunoperoxidase technique:

18 BPH and 19 CaP tissues were analysed for EGF receptors, as described under section 2.14.

Briefly, BPH and CaP cryostat sections were fixed in acetone for 20 minutes. After blocking endogenous interfering substances with normal rabbit serum (NRS), the sections were treated for 30 minutes with 1/30 dilution of monoclonal antibody (EGF-$R_1$). This was followed by secondary antibody (peroxidase conjugated rabbit antimouse immunoglobulin) and subsequent colour development with diaminobenzidine (DAB) and counter-staining with haematoxylin. Finally, sections were mounted with DPX.

Relationship between colour intensity and receptor level in BPH and CaP was established. Secondly, correlation between colour intensity due to receptor levels according to the histologic grades of the tumour based on Gleason score was established. Intensity was graded as negative (-), moderate (+) or very intense (++) - (+++).

Scoring was the result of 3 independent observers.

Plate 7: Showing BPH positive staining.

Plate 8: Showing well differentiated CaP stained section.

Plate 9: Showing poorly differentiated CaP stained section.

Plate 7 (x 133)

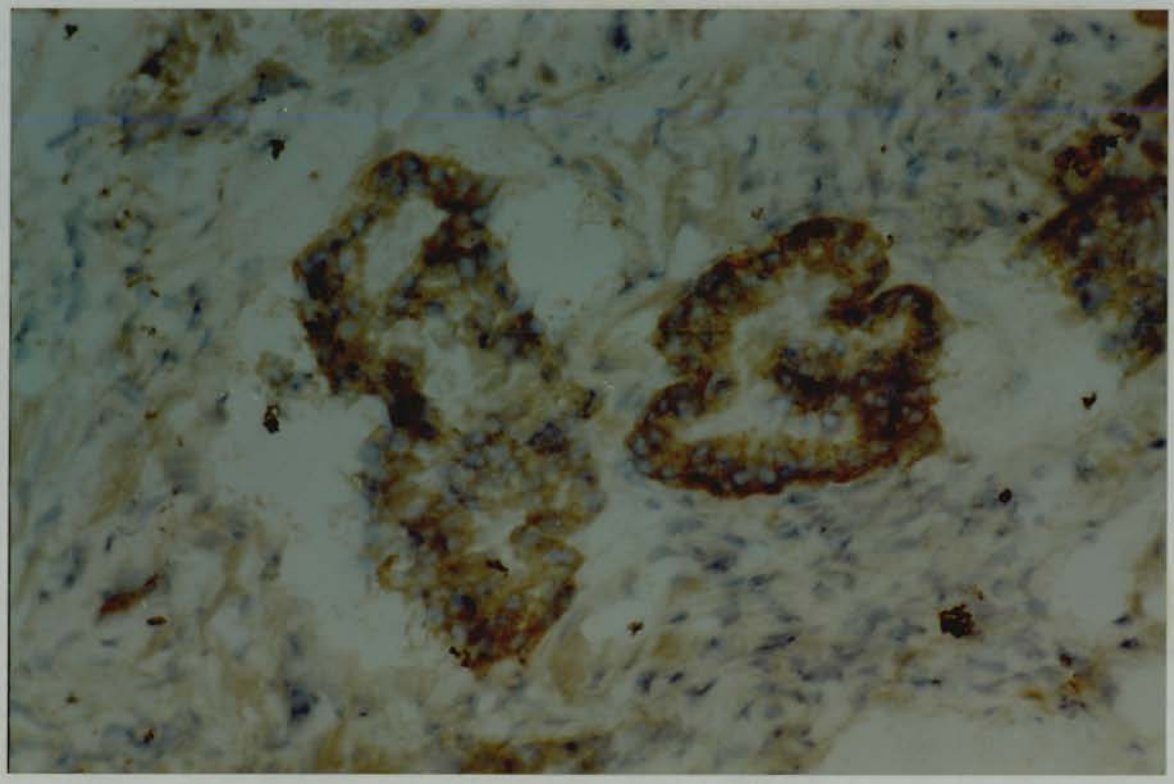


Plate 8 (x 333)

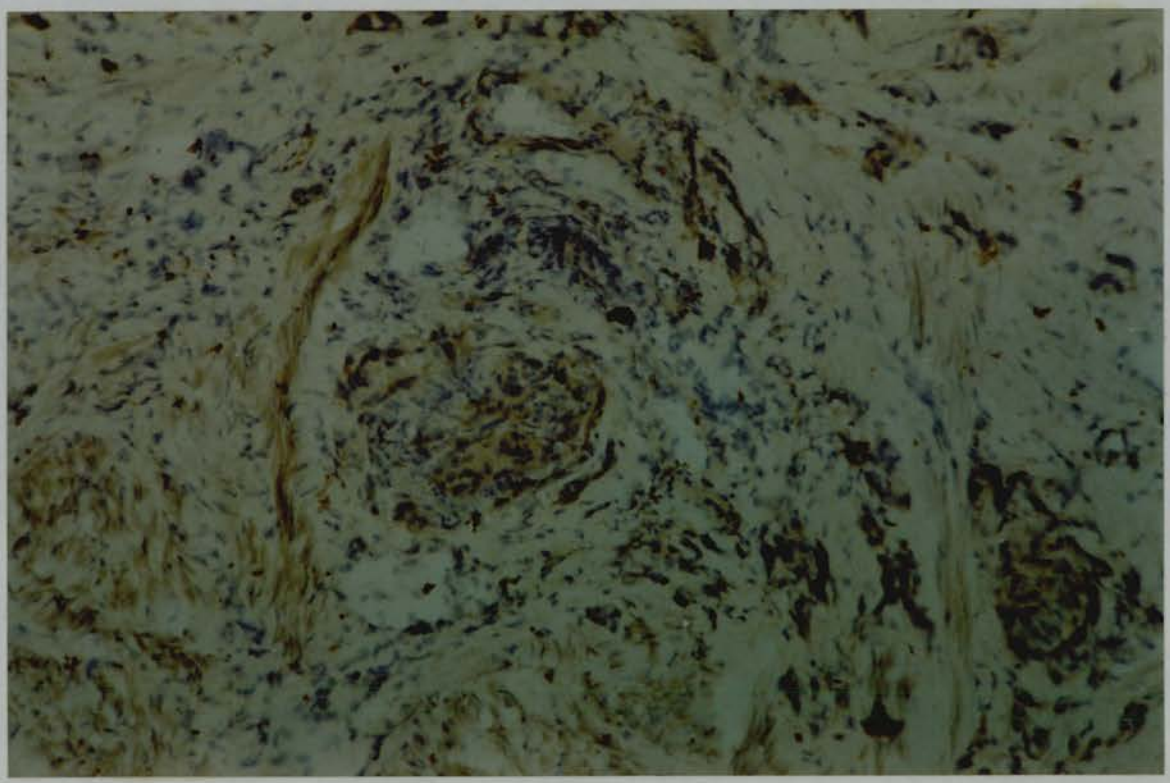


Plate 9A (x 133)

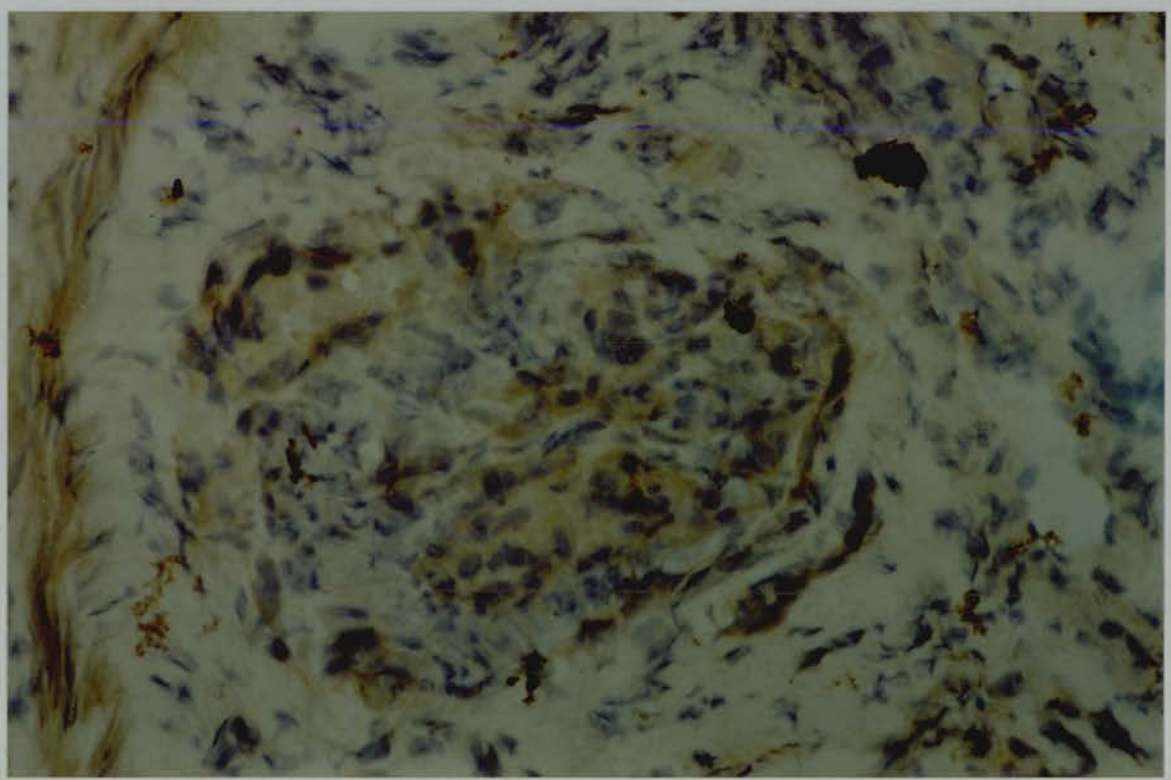


Plate 9B (x 333)

BPH (Plate 10) and well differentiated (WD) tumours (Gleason score 2 - 4) (Plate 11) demonstrated intense staining (+++), whilst poorly differentiated tumours (8 - 10) showed little or no staining (Plate 12). The WD tumours showed a closely packed acinar glands with little or no stromal invasion, whilst the poorly differentiated (PD) showed extensive stromal invasion. Good correlation was again observed between the immunocytochemistry and biochemical results.

Summary of radioligand assay and immunocytochemical results on BPH and CaP

Comparison between the immunocytochemical and biochemical results, as indicated in TABLE 5, reveals that there is good correlation between the two techniques. This correlation is not only seen in BPH tissues but also in CaP tissues. In the CaP intensity of staining varies according to the histologic grades of the cancer. These intensities also correspond to the receptor levels in (fmol/mg protein) measured biochemically, thus confirming good correlation between the results of the two techniques in all the 37 tissues analysed.

Plates 10, 11, 12: Presence of EGF receptors in BPH and CaP:

ii) Labelled avidin biotin technique:

The method is identical to the one described under section 2.14(b).

Briefly, frozen sections were treated with primary antibody EGF-$R_1$ (1/30 dilution) and incubated overnight. Sections were further treated with biotinylated sheep anti-mouse immunoglobulin followed by streptavidin - AP in the presence of alkaline phosphatase substrate (napthol ASBl phosphate).

Colour was developed by fast red ITR. Sections were subsequently counterstained with Mayers haematoxylin and finally mounted in glycerine jelly.

Plate 10: Showing BPH stained section.

Plate 11: Showing well differentiated CaP stained section.

Plate 12: Showing poorly differentiated CaP stained section.

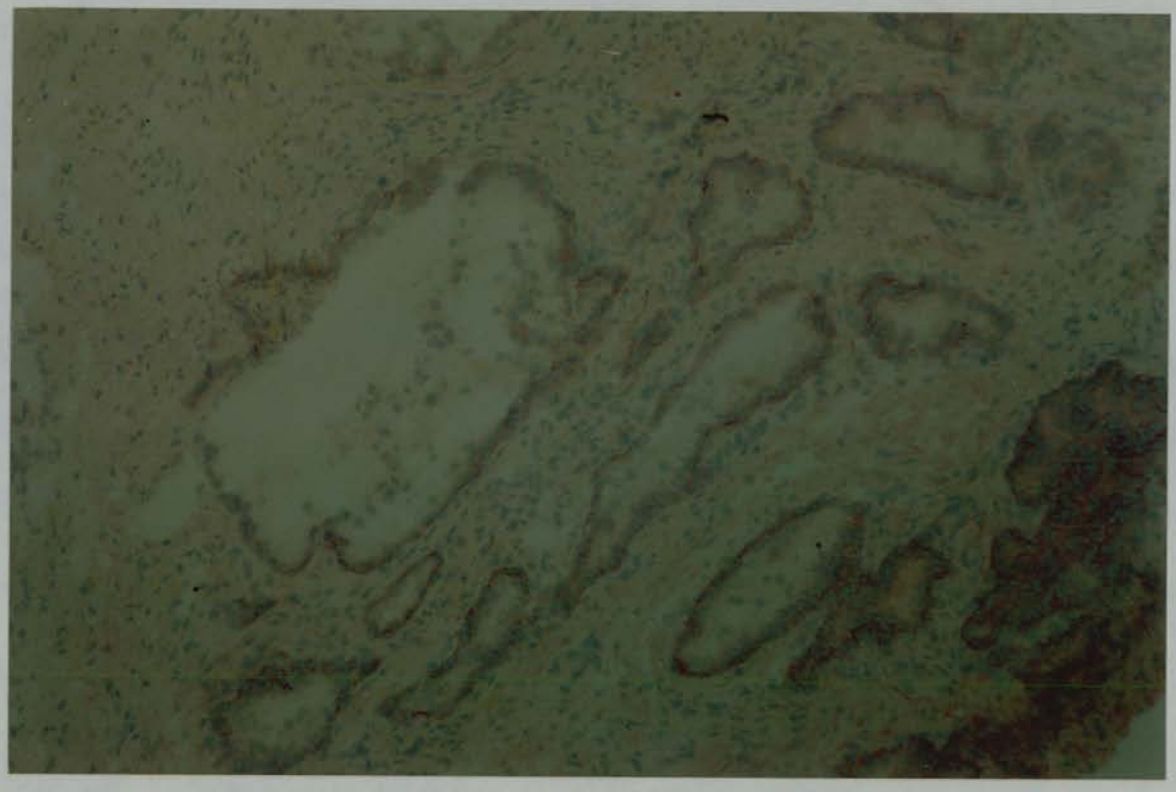


Plate 10 (x 133)

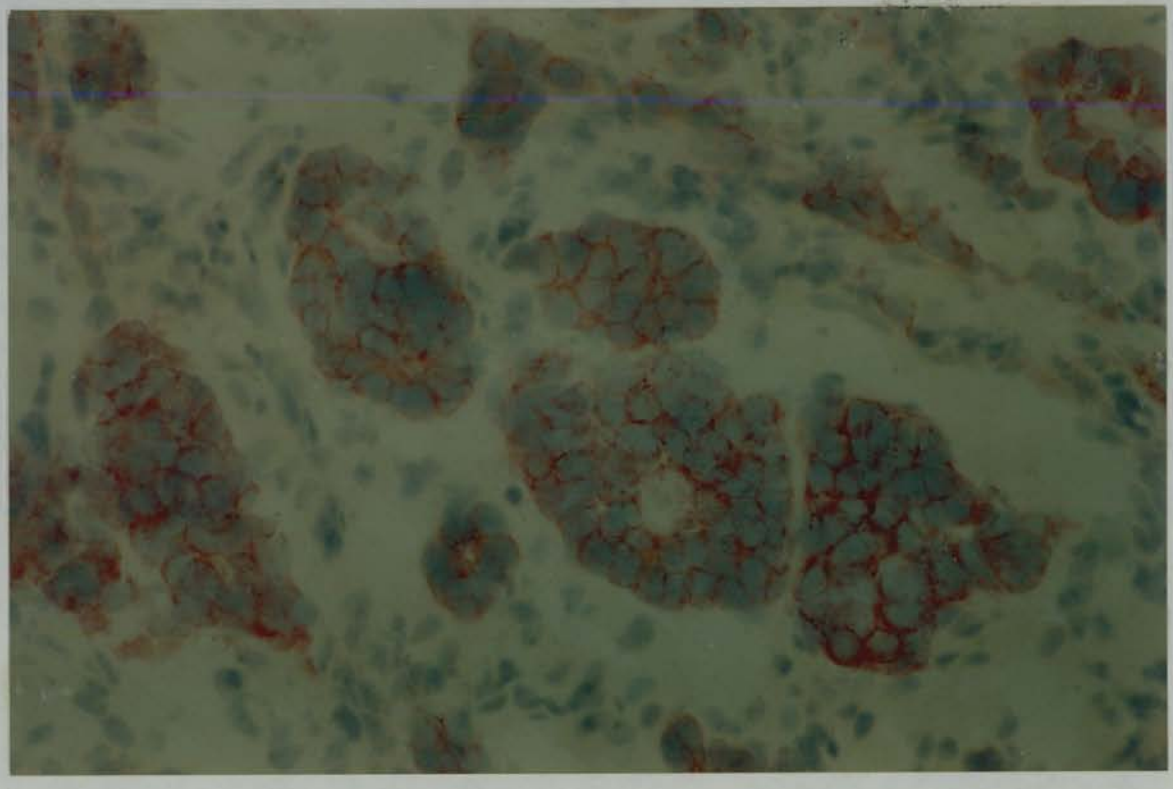


Plate 11 (x 333)

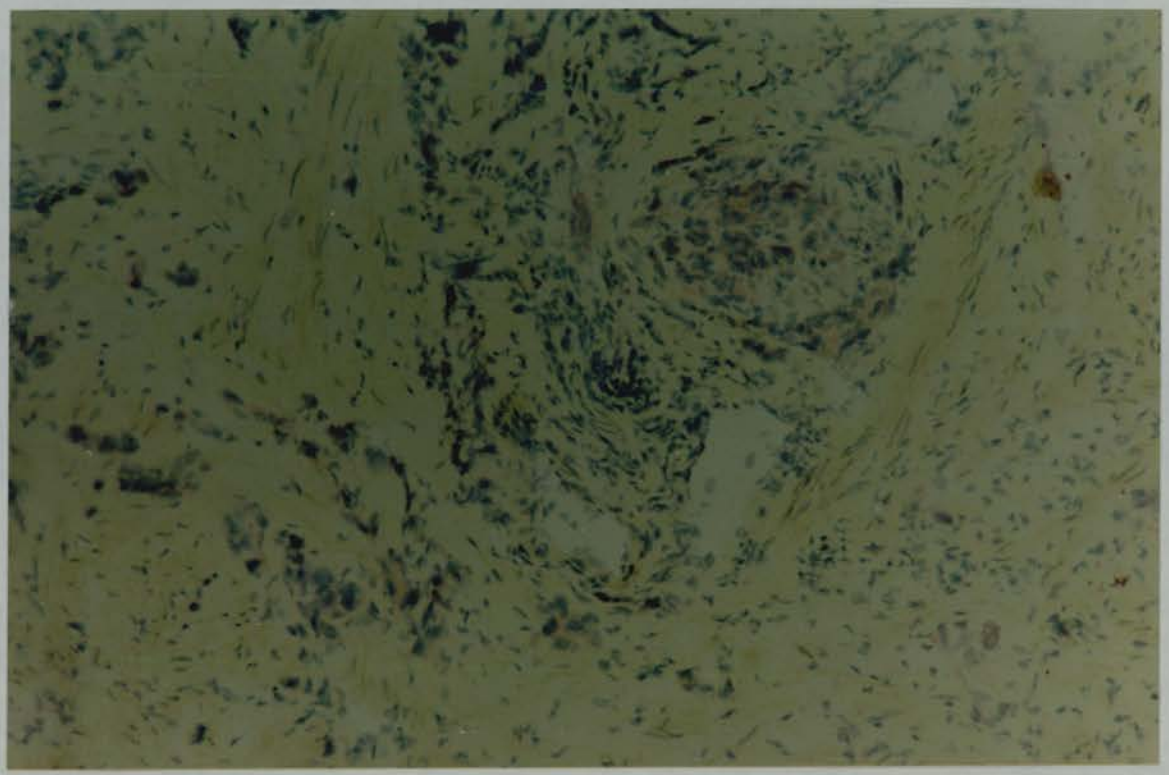


Plate 12A (x 133)

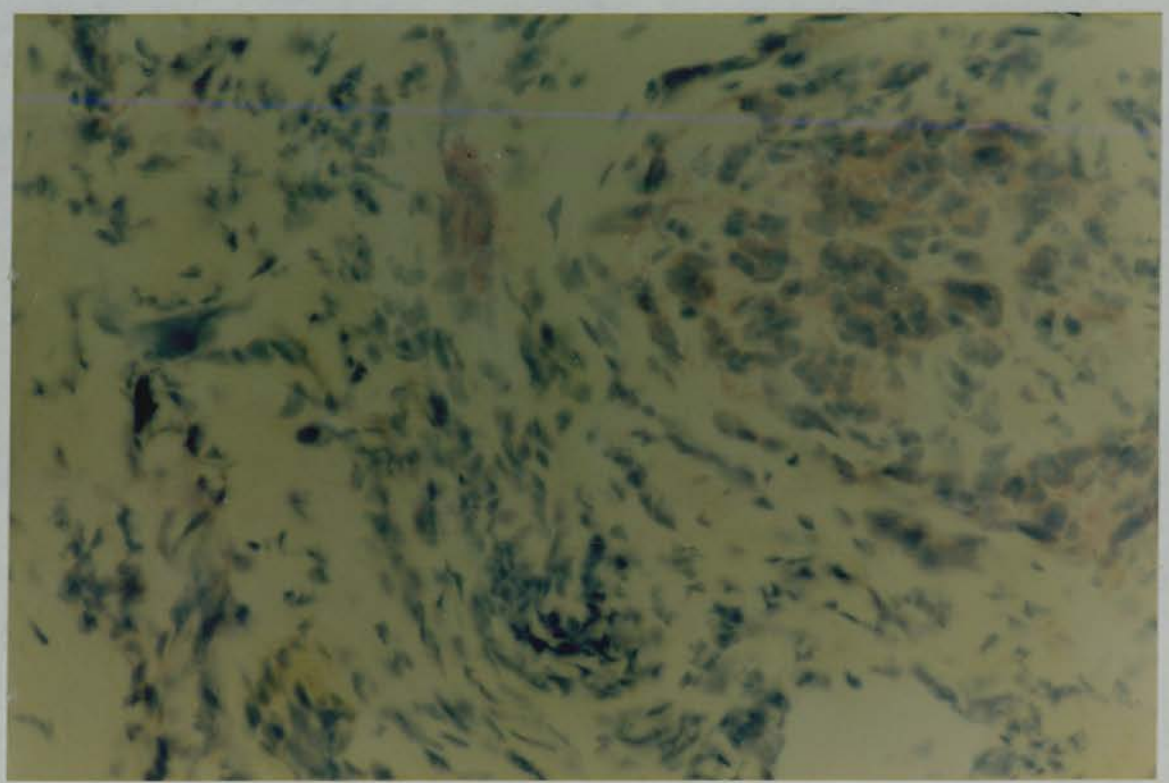


Plate 12B (x 333)

TABLE 5.

EGF RECEPTOR LEVELS IN BPH AND CaP:
SUMMARY OF RESULTS

Biochemical and immunocytochemical techniques were used to measure the EGF receptors in BPH and CaP tissues.

Details of the procedures are described under section 2.11 (Biochemical technique) and 2.14(a) and (b) (Immunocytochemical method). The results obtained for BPH using the two techniques and those obtained on various histologic grades of CaP are summarised. The biochemical results are expressed as number of binding sites in fmol/mg protein and as means $\pm$ SD of samples analysed.

The Immunocytochemical results are, however, expressed as scores of staining intensity and graded as negative (-), moderate (+) or intense (++) - (+++).

TABLE 5

| | GLEASON SCORE | MEAN S.B. fmols/mg PR. | STAINING INTENSITY | No STAINED |
|---|---|---|---|---|
| BPH | – | 125 | (+)–(+ + +) | 18 |
| CaP | 2–4 | 84 | (+)–(+ +) | 4 |
| | 5–7 | 52 | (+)–(+ +) | 10 |
| | 8–10 | 20 | – | 5 |

3.8 Demonstration of the presence of the internal domain of the EGF receptor using MAB F4:

This was investigated on 18 BPH and 19 CaP tissues by using a newly developed monoclonal antibody to the internal domain of the EGF receptor (F4) (Berger et al, 1987). In some tumours, loss of cell surface receptors is explained in terms of expression of truncated receptors which have no external domain and therefore are devoid of binding sites. The introduction of the F4 monoclonal antibody has enabled some workers (Berger et al, 1987) to look for truncated EGF receptors, i.e. the internal domain of the receptor in tumours. This prompted the author to look for the presence of truncated receptors or the internal domain of the EGF receptor in the poorly differentiated CaP which did not show any EGF binding, by using the monoclonal antibody F4.

Two immunocytochemical techniques were used, namely:-

a) Indirect immunoperoxidase technique

b) Labelled avidin-biotin technique

a) Indirect immunoperoxidase technique: MAB F4:

Staining with MAB F4, employing the above technique and using DAB as chromogen, produced an intense non-specific stromal staining.

As can be seen, Plates 13, 14, 15, 16 are stained sections of BPH, well differentiated and poorly differentiated CaP respectively, but the intensity of the stromal staining makes visualisation of the cytoplasmic staining produced by MAB F4 difficult.

b) Labelled avidin-biotin technique using monoclonal antibody MAB F4:

Staining with MAB F4, using the above technique, produced a much clearer picture. Staining was mainly cytoplasmic. No staining was associated with the basal layers of BPH (Plate 18).

Plates 13, 14, 15, 16

Demonstration of internal domain of EGF-$R_1$ using MAB F4 (Indirect immunoperoxidase method)

The technique is fully described under section 2.14(a).

Briefly, sections were fixed and treated with primary antibody (MAB F4) at concentration of 0.05 mg/ml for 30 minutes. Sections were thereafter passed through the whole staining procedure as outlined in section 2.14(a). Sections were later counter-stained with Harris haematoxylin and mounted using DPX.

Plate 13: Showing BPH stained section, negative control.

Plate 14: Showing BPH stained section, positive control.

Plate 15: Showing well differentiated CaP stained section.

Plate 16: Showing poorly differentiated CaP stained section.

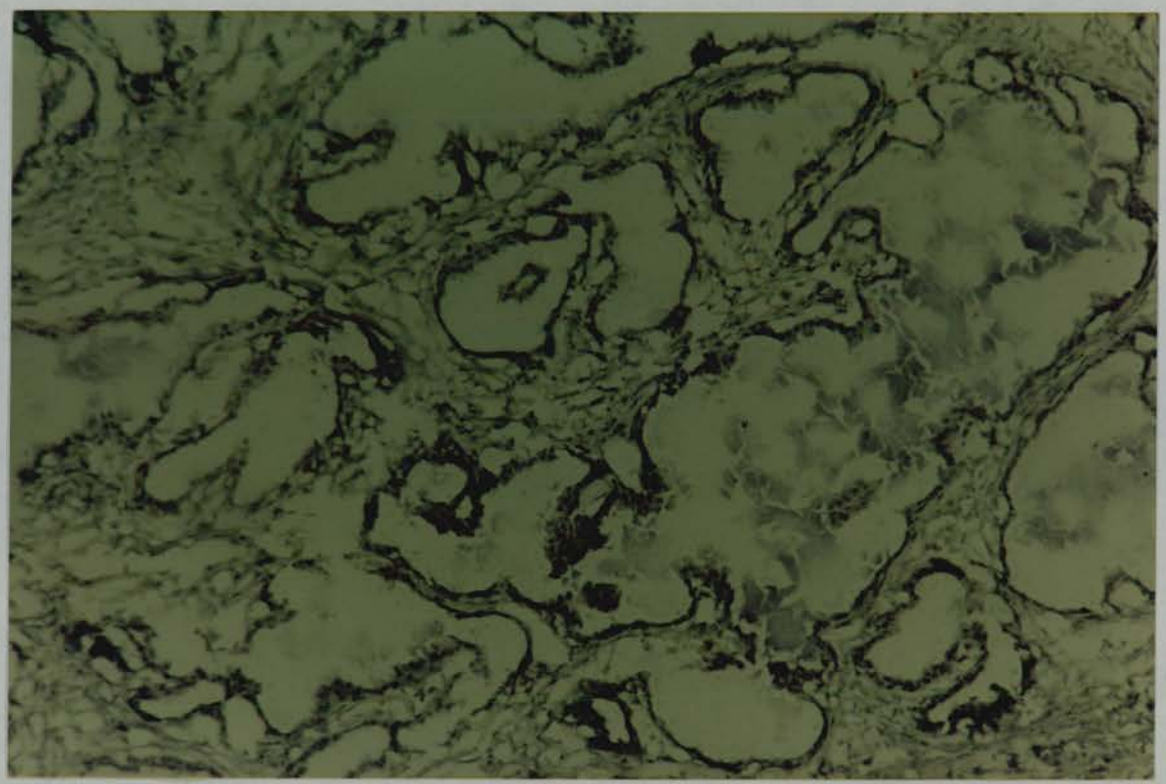


Plate 13 (x 133)

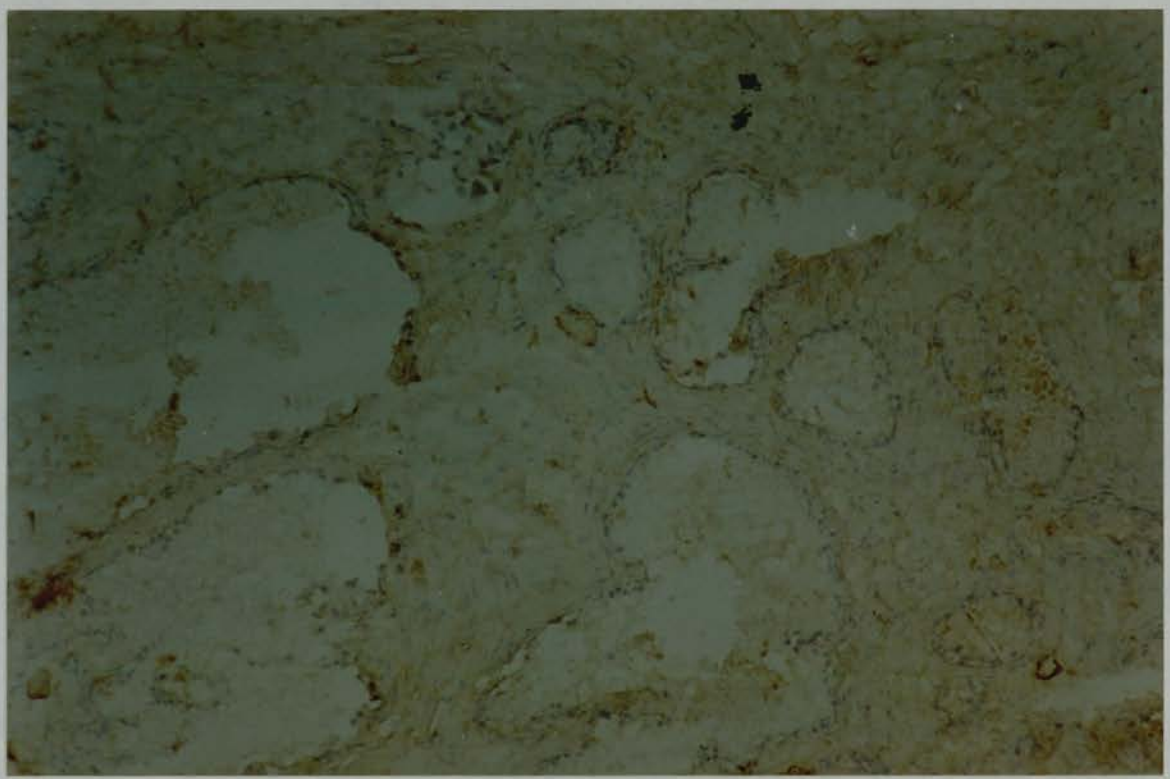


Plate 14A (x 133)

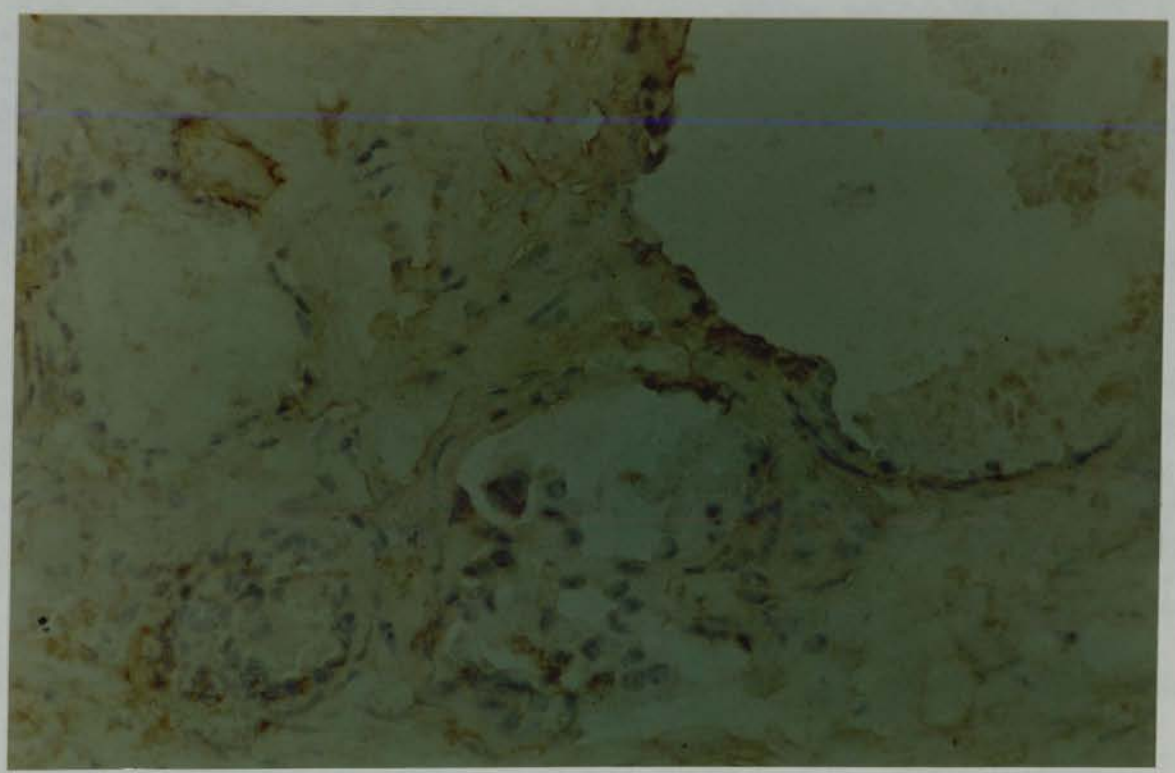


Plate 14B (x 333)

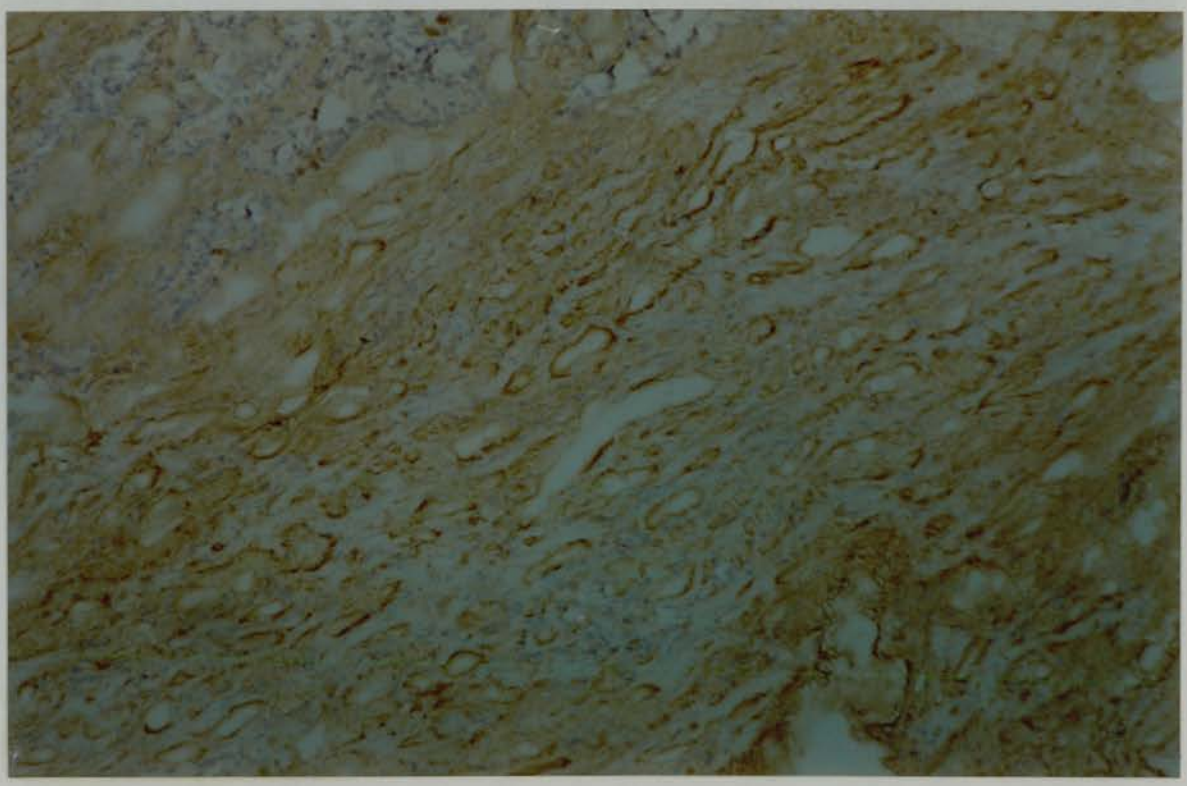


Plate 15A (x 133)

Plate 15B (x 333)

Plate 16A (x 133)

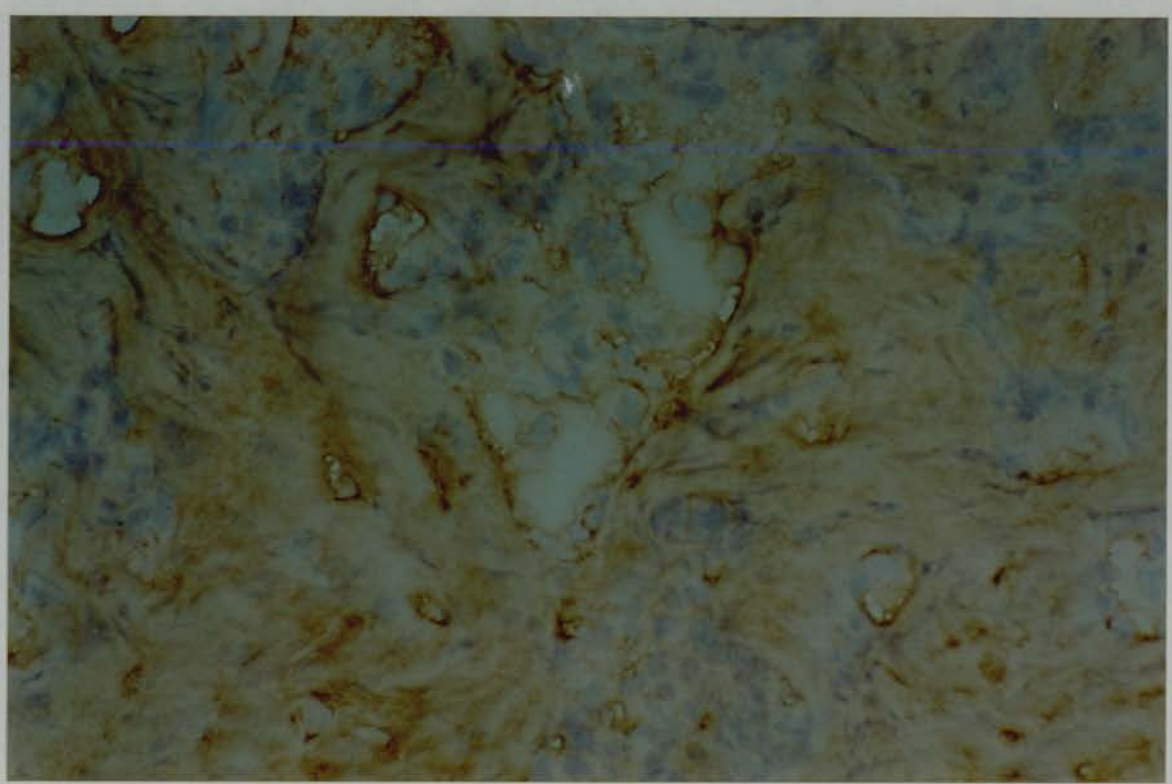


Plate 16B (x 333)

Well differentiated CaP (G/S 2 - 4) exhibited a much more intense staining (+++) (Plate 19), whilst the poorly differentiated CaP (G/S 8 - 10) was either weakly positive (+) (Plate 20) or was negative (Plate 17). It appears that the poorly differentiated CaP does not express any truncated receptors. Comparison of the intensity of staining produced by BPH (Plate 18) with that of well differentiated CaP showed that staining was more intense in CaP than BPH.

c) Comparison of staining results using monoclonal antibodies F4 and EGF-$R_1$

Immunocytochemical results on BPH and CaP using monoclonal antibody to the external domain (EGF-$R_1$) and the internal domain (F4) were compared.

TABLE 6 shows a summary of results obtained. On BPH the two monoclonal antibodies showed an intense staining: (++) - (+++) indicating the presence of both internal and external domains of the receptor in BPH, although the intensity of staining seen using EGF-$R_1$ was greater than that observed using the F4, but the staining intensity observed in the well differentiated CaP, using both EGF-$R_1$ and F4 was identical. The intensity was (+++) in each case. With regard to poorly differentiated CaP, monoclonal antibody EGF-$R_1$ did not show any staining at all. This picture was repeated using the F4 monoclonal antibody except an isolated patch which showed a faint positivity.

Furthermore, it is noteworthy that using the EGF-$R_1$, staining was confined to the basal layers of the epithelial cells of BPH, whilst the staining produced by F4 was mainly cytoplasmic. The absence of binding observed in the poorly differentiated CaP could stem from total disappearance of the receptors, probably with the external domain disappearing faster than the internal.

Plates 17, 18, 19, 20

Demonstration of internal domain of EGF receptor using MAB F4

The technique is described under section 2.14(b).

Briefly, frozen sections were fixed in acetone and passed through the staining procedure as outlined under section 2.14(b). Monoclonal antibody F4 was used as the primary antibody at a concentration of 0.05 mg/ml and incubation lasted overnight. Sections were subsequently treated with biotinylated sheep antimouse immunoglobulin followed by streptavidin - AP in the presence of alkaline phosphatase substrate. Colour was developed by fast red ITR. Sections were counter-stained with Mayers haematoxylin and finally mounted in glycerine jelly.

Plate 17: Showing BPH stained section, negative control.

Plate 18: Showing BPH stained section, positive control.

Plate 19: Showing well differentiated CaP stained section.

Plate 20: Showing poorly differentiated Cap stained section.

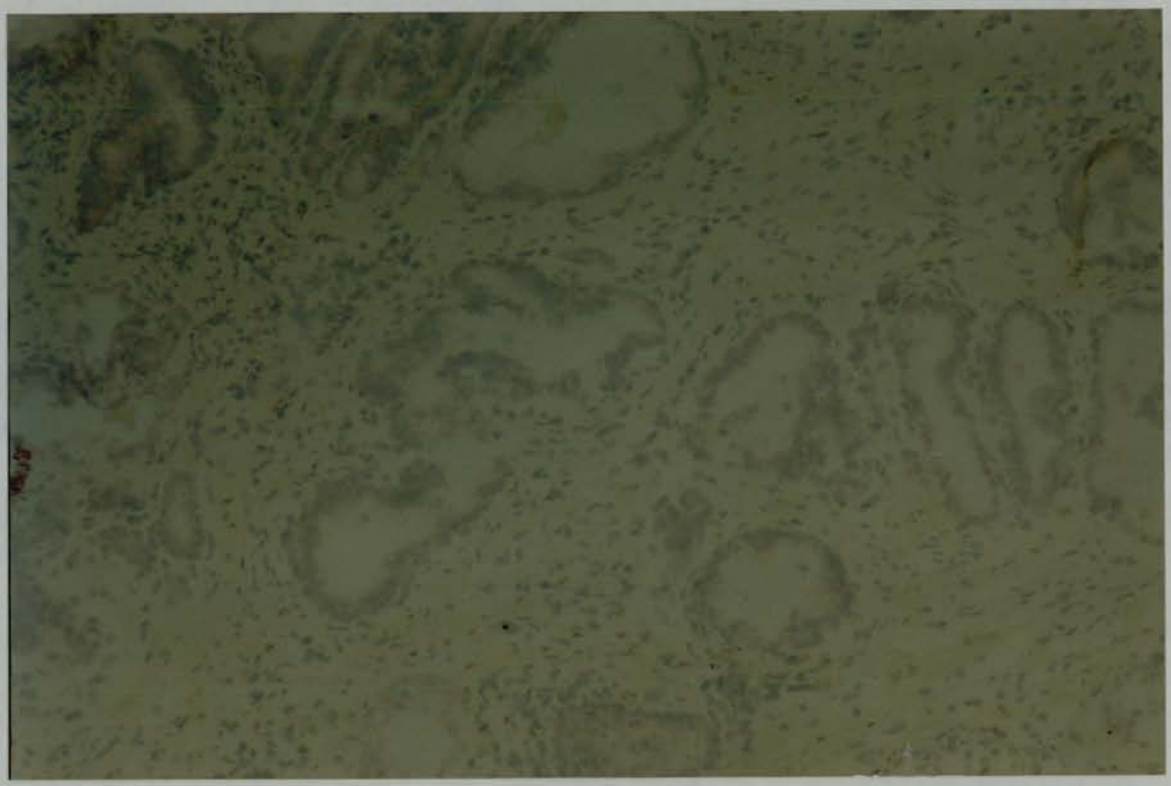


Plate 17A (x 133)

Plate 17B (x 333)

Plate 18A (x 133)

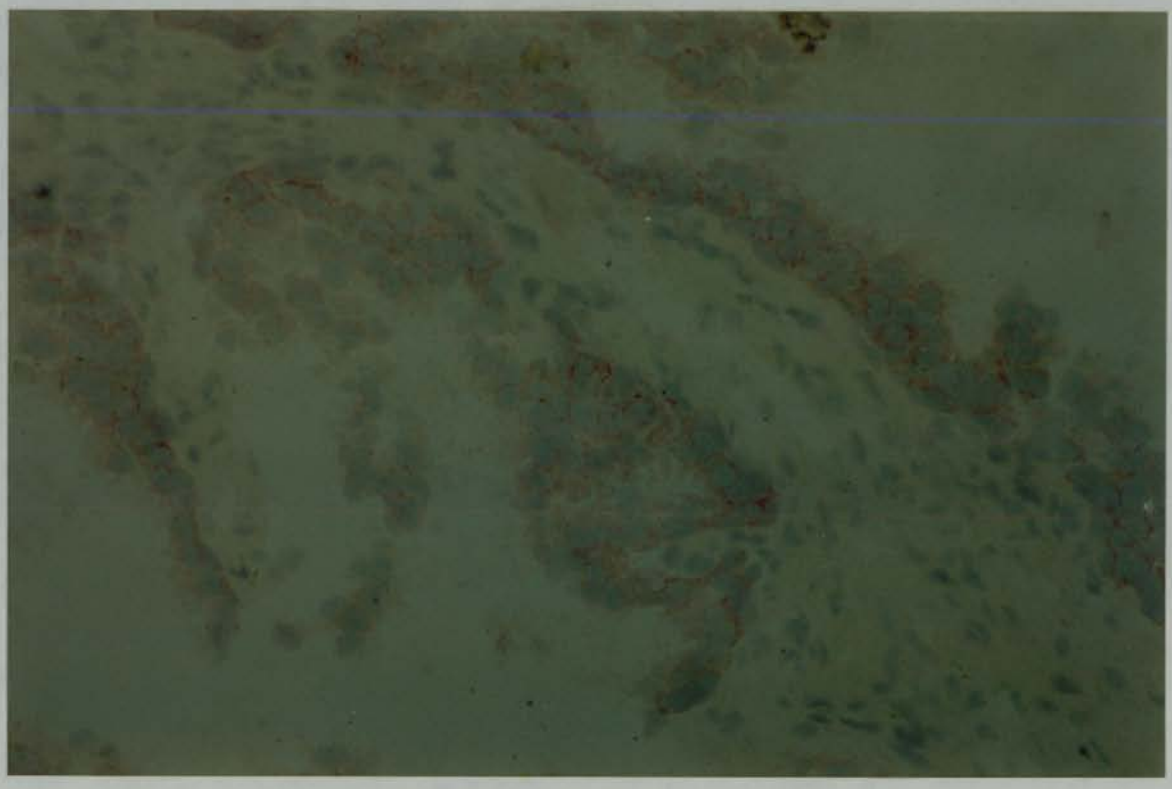


Plate 18B (x 333)

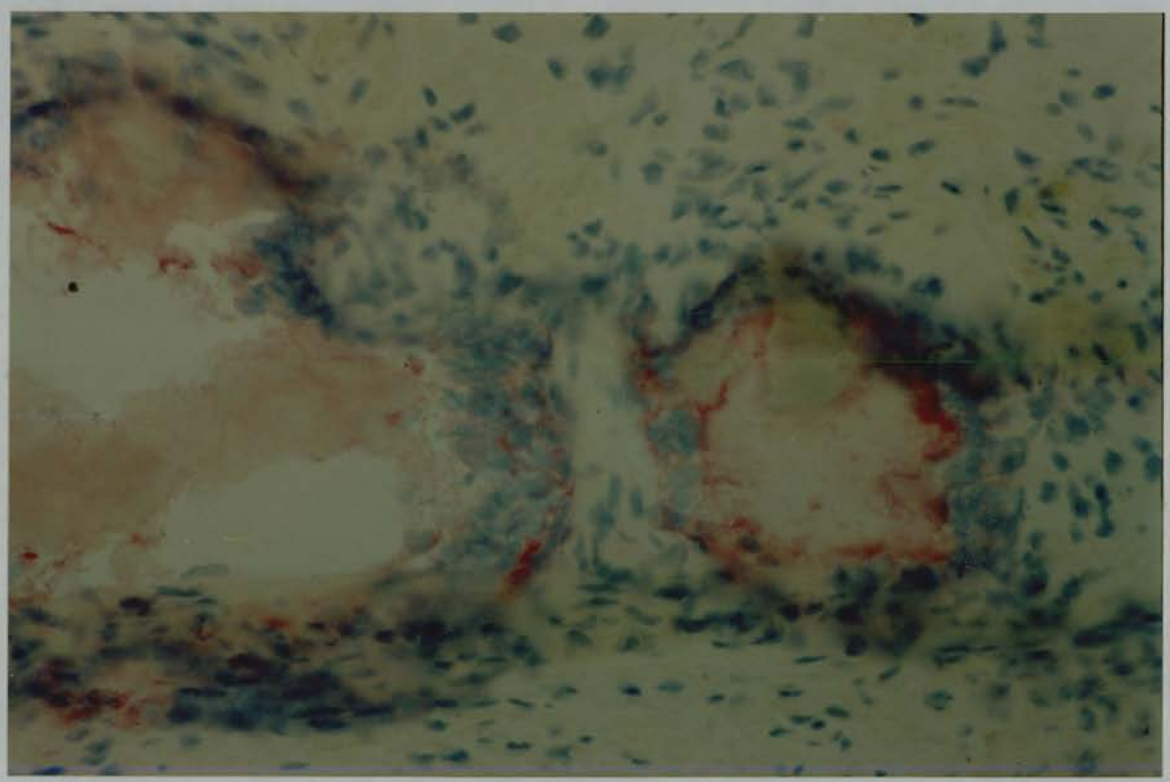


Plate 18C (x 333) is also showing secretions into the acinar.

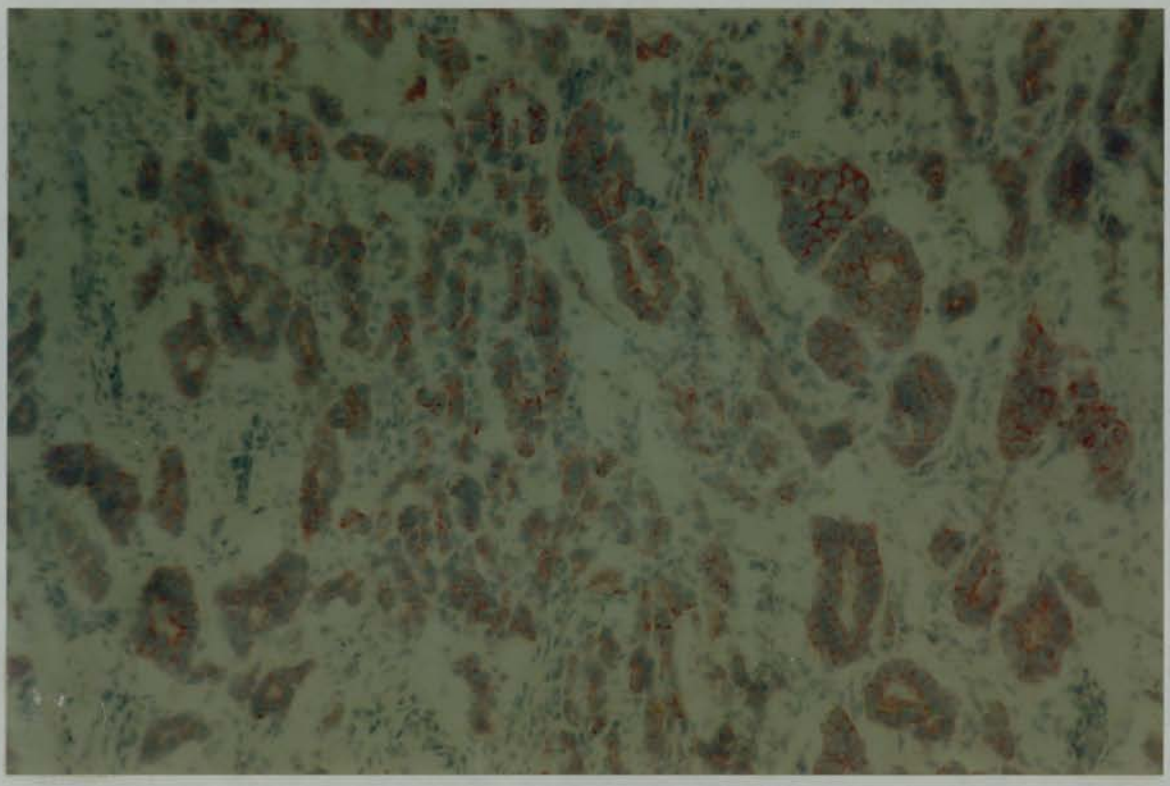


Plate 19A (x 133)

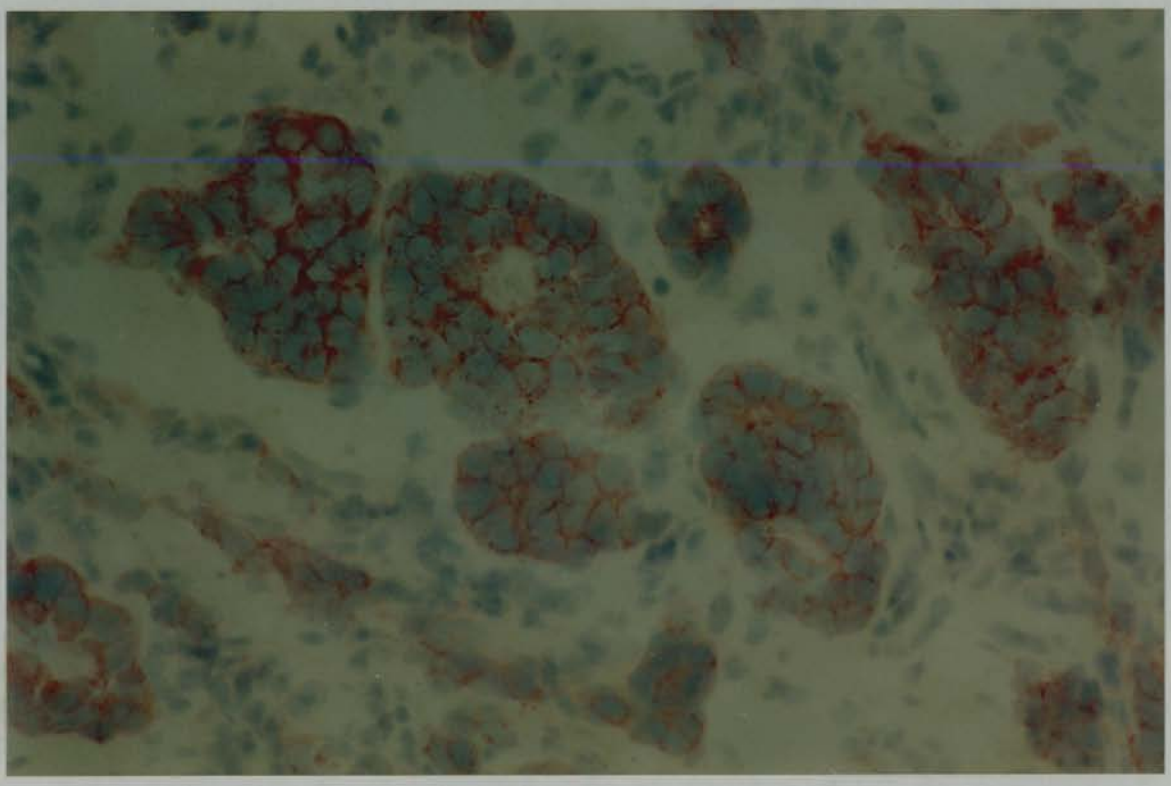


Plate 19B (x 333)

Plate 20A (x 133)

Plate 20B (x 333)

TABLE 6.

SUMMARY OF STAINING RESULTS USING MONOCLONAL ANTIBODIES EGF-$R_1$ AND F4

Labelled avidin biotin staining technique was used to demonstrate the presence of the external and internal domains of the EGF receptor using monoclonal antibodies EGF-$R_1$ and F4 respectively.

The technique is identical to the one described under section 2.14(b).

Briefly, frozen sections were treated with primary antibodies EGF-$R_1$ (1/30 dilution) and F4 (0.05 mg/ml concentration) and incubated overnight. Sections were further treated with biotinylated sheep antimouse immunoglobulin followed by streptavidin - AP in the presence of alkaline phosphatase substrate (napthol ASBl phosphate). Colour was developed by fast red ITR. Sections were subsequently counterstained with Mayers haematoxylin and finally mounted in glycerine jelly.

The procedure was performed on BPH, well (G/S 2 - 4) and poorly differentiated CaP (G/S 8 - 10) tissues. Staining intensity was scored as negative (-), moderate (+) or intense (++) - (+++) by 3 different observers. The staining localisation and intensity obtained by each monoclonal antibody for each tissue type is summarised.

TABLE 6.

| Tissue | EGF-$R_1$ (Ext. Domain) | $F_4$ (Int. Domain) |
|---|---|---|
| B.P.H. | +++ | ++ |
| Ca P (W.D.) | +++ | +++ |
| Ca P (P.D.) | - | +- |
| Area stained | Basal layers of epithelium | Cytoplasmic |

Other experiments:

3.9 EGF receptor blocking experiment:

Radiolabelled monoclonal antibodies are now widely used in immunotherapy. Increased expression of EGF receptors on human glioblastomas prompted Epenestos et al (1985) to treat a patient with glioblastoma multiforme which was EGF receptor positive, with monoclonal antibody to the receptor, labelled with iodine 131. In view of these reports, it was decided to investigate if the monoclonal antibody could block the EGF binding with the human prostate.

Comparison of the data presented in TABLE 7 indicates that pretreatment of the prostate particulate fraction with monoclonal antibody did not achieve any blocking effect (A). EGF binding studies still showed high levels of specific binding as control (B).

Similarly, pretreatment of sections with EGF prior to immunocytochemistry still showed positive staining. Plate 21 is showing staining before treatment whilst Plate 22 indicates staining after treatment.

These results show that the two treatments could not achieve any blocking effect. The reason for these results may be due to the fact that EGF may be recognising a site distal to the binding site of the monoclonal antibody.

TABLE 7.

BLOCKING OF EGF RECEPTOR SITE

BPH particulate fraction was pretreated with 1/30 dilution of monoclonal antibody to the EGF receptor site (external domain) (Column A).

After washing the particulate fraction with buffer B and centrifugation, pellet was reconstituted in 500 µl buffer B. 100 µl aliquots of the treated sample were incubated with 100 µl of $^{125}$I-EGF in the presence and absence of 50-fold excess unlabelled EGF at 37$^{o}$C for 90 minutes. $^{125}$I-EGF bound complex was separated from free by PEG precipitation and centrifugation. Specific binding was calculated by the method described under section 2.11.

In another experiment the above procedure was repeated, on the same particulate fraction, which was not treated with monoclonal antibody and this served as the control (Column B).

The test was repeated on 3 different samples and results are expressed as means $\pm$ SD.

TABLE 7.

| TEST A | CONTROL B |
|---|---|
| WITH MONOCLONAL ANTIBODY PRETREATMENT | WITHOUT MONOCLONAL ANTIBODY PRETREATMENT |
| MEAN SPECIFIC BINDING = 707 dpm ± 20 | MEAN SPECIFIC BINDING = 703 dpm ± 30 |

Plates 21 and 22

EGF receptor blocking experiment
Immunocytochemical technique
(Indirect immunoperoxidase method)

The method is outlined under section 2.14.

Briefly, 3 - 4 um sections were fixed in acetone and pretreated with EGF (300 ng/ml). Another section was not pretreated with EGF and this served as control. The two sections were subjected to the same steps outlined in 2.14(a) using monoclonal antibody to the external domain of the EGF receptor.

Plate 21: Before treatment.

Plate 22: After treatment.

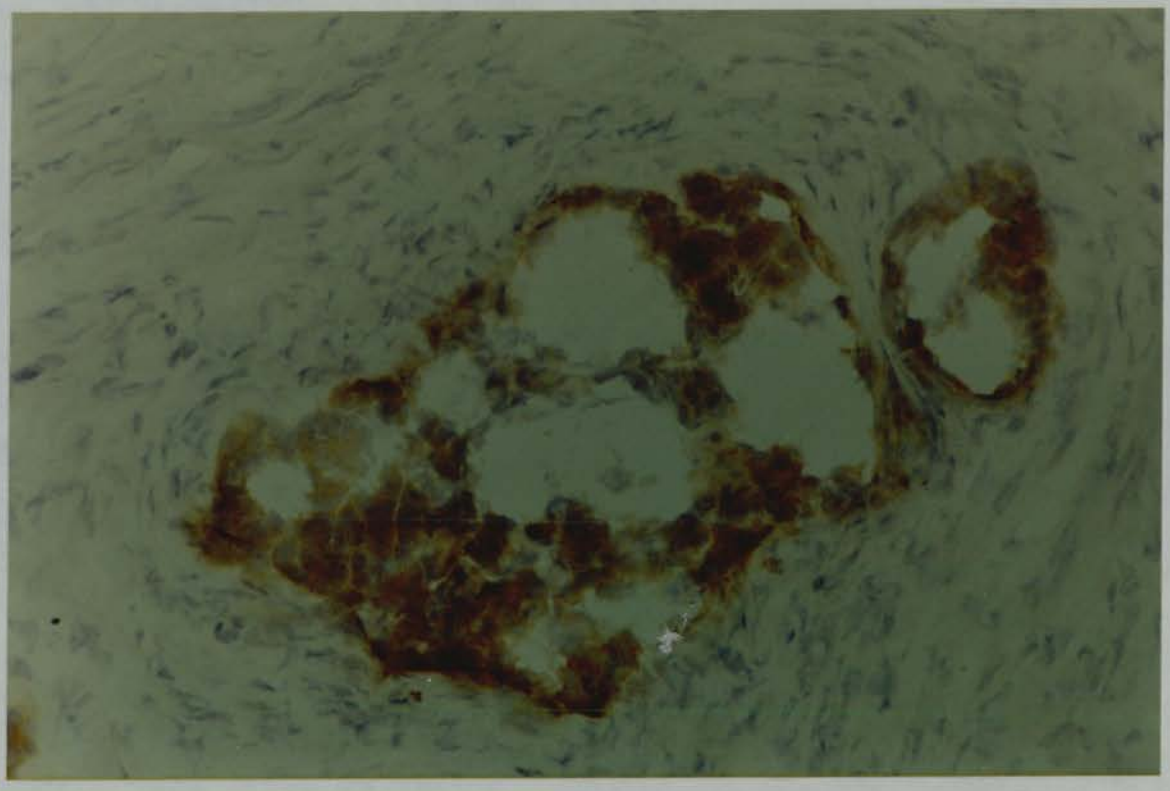


Plate 21 (x 333)

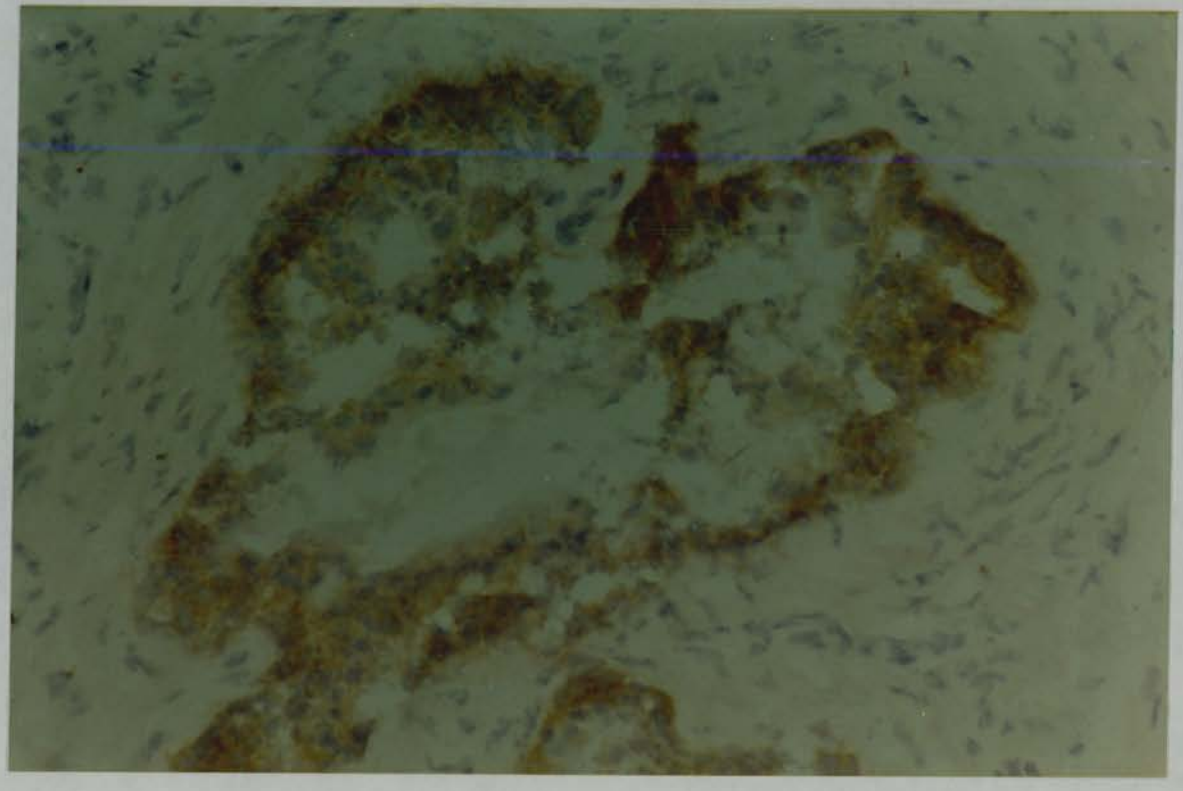


Plate 22 (x 333)

# CHAPTER 4

# DISCUSSION

## CHAPTER 4. DISCUSSION

Data presented in this report demonstrate for the first time that the human prostate contains high affinity binding sites for mEGF. Biochemical and immunocytochemical techniques show that more than 85% of BPH tissues examined contain the EGF receptors which are found to be mostly concentrated in the basal layers of the epithelial cells.

Prior to characterisation of the binding proteins in BPH, it was decided to study the pattern of EGF binding with subcellular fractions of BPH tissue. Since no enzyme markers were used, these fractions should not be described as pure. Furthermore, methods of separating specific from non-specific binding were also investigated.

### 4.1 BINDING WITH SUBCELLULAR FRACTIONS: SEPARATION METHODS

Investigation into EGF binding with subcellular fractions of BPH, demonstrated the pattern of distribution of the receptors among the intracellular organelles. This revealed that the bulk of the receptors (68%) are associated with the 800g crude heavy pellet, whilst the remaining receptors were distributed between the microsomal and mitochondrial fractions. There was no specific binding associated with the cytosol fraction (TABLE 3). The presence of the ligand binding sites in the intracellular organelles is difficult to explain. Ramani et al (1986) made similar observations when they studied the distribution of EGF receptors in the human placenta organelles. The following are some possibilities which may explain the presence of the receptors in the organelles. It is thought that receptors in the organelles might represent those in a catabolic route following internalisation. Binding with exogenously added $^{125}$I-EGF suggested that the endogenous bound ligand dissociated during the experimental procedure or during internalisation process. Furthermore, receptors in the intracellular organelles might represent newly synthesised receptors. A portion of the intracellular receptor might be a normal

component of the organelles. Internalised $^{125}$I-EGF might associate with the intracellular organelles leading to generation of biological signals of the ligand action. The final fate of the receptor bound complex, however, is not clear, but it is thought that internalised receptors are either recycled or degraded (Stoscheck and Carpenter, 1984; Teslenko et al, 1987).

It is not certain whether the EGF binding proteins associated with the various subcellular fractions of the BPH homogenate have identical characteristics or are different proteins. Although Ramani et al (1986) observed that all the EGF binding proteins associated with the subcellular fractions of the placenta exhibited curvilinear plots, these proteins also showed other different biochemical characteristics. Similar detailed investigation may help to establish fully the binding characteristics of the proteins which are associated with the various fractions of BPH.

Alternatively, since marker enzymes were not used to ensure the purity of the subcellular fractions, it is also possible that $^{125}$I-EGF binding associated with the various fractions could have resulted from cross-contamination from one fraction to the other. In this regard, it is possible that all the binding proteins could be identical.

The importance of separating bound complex from free during $^{125}$I-EGF binding studies cannot be overemphasised. The reliability and reproducibility of the binding assay depend in part on a very efficient method of achieving separation between the specific and non-specific binding.

Experiments designed to select a suitable method of separating specific from non-specific binding show that PEG precipitation and centrifugation technique is the most suitable method for achieving

this effectively. Comparison between filtration and PEG methods (Figure 12) shows that the PEG method produced 16% more specific binding than the filtration technique compared to control values. Furthermore, the background count for the PEG method is also 16% less than that of the filtration technique, provided the centrifugation time of the PEG method is restricted to 20 minutes (Figure 14), which time has been shown to be optimal for EGF binding in BPH. Centrifugation for less than 20 minutes (Hwang et al, 1986) may have undesirable effects on the binding and this may include underestimation of the EGF binding in BPH.

## 4.2 CHARACTERISATION OF THE EGF RECEPTOR IN BPH

The features of EGF binding in BPH are similar to those observed for other EGF target tissues. EGF binding in BPH is time and temperature dependent (Figure 16). The dissociation of bound complex is also time dependent, being faster in the presence than absence of excess unlabelled EGF (Figure 17A and B). The EGF receptors are saturable (Figure 18 (insert).

The specificity of binding is also demonstrated by the fact that no other unlabelled polypeptide exhibited any competition apart from mEGF (Figure 19), although slight competition (< 20%) was offered by human growth hormone, probably indicating a role for growth hormone in the prostate. Scatchard plot analysis has revealed a curvilinear plot suggestive of two classes of binding sites of higher dissociation constant (Kd) (mean = $0.8 \pm$ SD 0.2 nmol/L) and lower dissociation constant (Kd) (mean = $7.6 \pm$ SD 2.8 nmol/L) affinities (Figure 18). Curvilinear plots have also been reported by many workers including Hofmann et al (1984); Sainsbury et al (1985) and Ramani et al (1986). Although the lower affinity values differ from one target tissue to the other, it is noteworthy that the higher affinity values are all confined to

a range of 0.2 nmol/L to 2 nmol/L. In this regard, the value for the higher affinity receptors obtained in this report (0.8± .2 nmol/L) falls within the usual range reported by other workers. Furthermore, the workers who reported linear Scatchard plots consistent with single class of binding sites (Hock+Hollenberg, 1980; Mukku+Stancel, 1985; Traish +Wotiz, 1987) also obtained only the higher affinities in the range 0.2 - 2 nmol/L. This range of affinities appears to be part of the general characteristics of the EGF receptor. It is therefore in order to conclude that these characteristics also apply to the EGF binding protein in the human prostate.

The presence of curvilinear Scatchard plots has been explained in many ways. Curvilinear plots are suggestive of multiple binding sites resulting from heterogenous nature of the tissue, under investigation. In a recent study on whole mammary glad membranes, two sets of independent receptor sites were identified (Edery et al, 1985), whereas only one class of receptors was demonstrated in isolated epithelial cells from the same gland. Since the prostate also consists of a mixture of epithelial and stromal cells (Cowan et al, 1977) it is conceivable that the heterogeneity of the specimen analysed could be responsible for the curvilinear Scatchard plots obtained in this study, but the localisation of the EGF receptors to the basal layers of the epithelium (Plate 2) makes this possibility unlikely. The significance of the two binding sites is not well understood. In this report two forms of protein have been described (Figure 15) and whether the curvilinear plots observed stem from the presence of the two different proteins is not very clear. However, recently Boni-Schnetzler+Pilch, (1987) showed that the presence of high and low affinity binding sites was the result of formation of active receptor dimers from inactive

receptor monomers. Active dimer formation was thought to be necessary for the generation and maintenance of the mitogenic signal. Furthermore, it is believed that only a small portion of the receptors bind with high affinity and a large portion bind with low affinity, as seen in this report (Figure 18). Other evidence for the existence of two forms (higher and lower) affinities of EGF receptors, was given by Adamson + Rees (1981) who suggested that EGF bound preferentially to the higher affinity and later to the spare or lower affinity receptors. The lower affinity receptors have recently been implicated in inhibition of cell differentiation (Boonstra et al, 1985).

Investigation of effect of tissue protein concentration on EGF binding in BPH shows that there are two forms of EGF binding proteins (Figure 15 A and B). It appears that one form of protein is present at a lower concentration up to 1 mg/ml (Figure 15A) whilst the second form is present at a much higher concentration, between 1.5 and 8 mg/ml (Figure 15B). This probably explains in part the two linearities seen (0.1 - 1 mg/ml) and (1.5 - 8 mg/ml). Furthermore, the presence of two forms of protein is again demonstrated when BPH particulate fraction is exposed to higher temperature prior to EGF binding. Exposure of the particulate fraction at 45°C for 10 minutes prior to binding reversibly inactivates the EGF receptor by 90% of control, as shown by the binding result (Figure 21A), but exposure of the fraction at higher temperatures of 65°C and 75°C for 10 minutes paradoxically produced 30% and 40% increased binding respectively over control value (Figure 21A). However, when the particulate fraction was exposed to 95°C for 10 minutes prior to binding there was a complete irreversible inactivation of the receptor, as shown by the binding result (Figure 21A). But incubation of the fractions at 65°C, 75°C

and 95°C show that the rise in binding observed for 65°C and 75°C is only transient, as it falls sharply after 30 minutes and 20 minutes respectively. At 37°C (control) however, no such fall is observed, whilst at 95°C no binding is observed at all (Figure 21B).

This finding indicates that there must be two forms of EGF binding protein present in BPH, and that one form is more sensitive to heat than the other. Alternatively, the transient increase of EGF binding observed at the higher temperatures may represent occupied endogenous receptors which are dissociated from the EGF receptor complex at a higher temperature and therefore bind exogenous EGF. The tightness of the complex may be so strong that it may require a higher temperature to dissociate it. EGF is known to be heat-resistant up to 100°C for 10 minutes (Taylor et al, 1974), but not much is known about the effect of heat on the EGF receptor. Hock and Hollenberg, 1980; Ramani et al (1986), however, observed irreversible inactivation of the EGF receptor at 55°C and 65°C respectively in the human placenta.

The literature is also full of information about proteins which are formed as a result of heat shock. Whether one of the two forms of proteins seen in the two experiments described above (Figures 15 A and B; 21 A and B) belong to this category of heat shock proteins is not clear. The response of cells to heat shock has been known since the work of Ritossa (1962), on Drosophila embryos. Studies on heat shock proteins (HSPS) have been carried out by many workers including Kelly + Schlesinger (1978). It is now known that virtually all organisms from E. Coli to man have heat shock proteins (Schlesinger et al, 1982). Major heat shock proteins were thought to have been strongly conserved in structure through evolution, indicating that

they play a role in the survival of the organism. Their presence appears to enhance the cell's ability to recover from stress, but precisely how is the question many workers are trying to answer. The molecular weight of heat shock proteins is thought to be small, being in the range of 20 - 90 KDA. It is believed that the small molecular weight heat shock proteins form large insoluble aggregates after heat shock, but these aggregates dissociate during cell recovery (Collier and Schlesinger, 1986).

Cells from different organisms respond differently, depending on the extent of the temperature stress. This may probably explain the difference in heat response by the placenta (Hock+Hollenberg, 1980; Ramani et al, 1986) and the prostate (Figure 21) in this report.

Serendipity is the gift of finding valuable or agreeable things not sought for, and it accounts for many important discoveries. The heat shock phenomenon is no exception. The way heat shock proteins help a cell tolerate heat remains a mystery. It has been assumed that the consequence of heat shock is always denaturation of proteins followed by degradation, but it has now been shown that this is not always the case. Munro+Pelham (1984) showed that there was stabilisation of proteins after heat shock.

Earlier reports indicated absence of EGF receptors in the human BPH tissue (Gregory et al, 1986). This prompted investigation into some of the possible factors that could influence EGF receptor levels in BPH.

Comparison of urogastrone binding, with the binding of mEGF to the human BPH particulate fraction showed that the level of specific binding produced by mEGF was 70% more than what was observed for urogastrone (TABLE 4). This means that using urogastrone, the level

of EGF receptor in BPH is grossly underestimated by as much as 70%. This degree of underestimation could have important implications on the EGF receptor measurement, particularly when EGF receptor levels are low in the tissue. Low result could also be further accentuated if microsomal fractions are used instead of total particulate fraction (TABLE 3).

Although the above report confirms the findings of Savage et al (1972) and Gregory (1975), the degree of binding to BPH by each ligand is found to be very different. The difference in the binding characteristics of the two ligands may be due to their different affinities for EGF receptor in BPH. Since the work of Gregory et al (1986) involved interaction between $^{125}$I-urogastrone and the human BPH, it is tempting to speculate that the absence of receptors obtained could be due to low affinity of human urogastrone for BPH and probably the use of microsomal fraction.

The prostate tissue was further investigated for the presence of endogenous EGF or its receptor. The use of $MgCl_2$ and dextran coated charcoal (DCC) failed to establish the presence of endogenous EGF in BPH (Figure 24A). The absence of endogenous EGF in the human prostate had been previously established (Elder et al, 1978; Gregory et al, 1986) using immunocytochemical methods. The absence of endogenous EGF in the rat prostate was also recently established using $MgCl_2$ and DCC, but Leake et al (1983), on the other hand, were able to improve prolactin binding in the human prostate using these reagents. $MgCl_2$ has been shown to dissociate tightly bound endogenous ligands from their receptors, whilst DCC removes possible binding inhibitors from the tissue extracts (Kelly et al, 1979). However, Hirata and Orth (1979) measured a small quantity of EGF (less than 1ngEGF/g) from

the prostate of a 48 year old man obtained at autopsy, using radio-immunoassay technique. Similarly, Traish+Wotiz (1987) also measured large quantities of EGF (5 - 10 ng/g) in the rat prostate, using a radio-immunoassay technique based on a commercial kit.

These reports showed that the choice of technique had important implications on detection or measurement of endogenous ligand or its receptor. Furthermore, on the basis of these reports, it would seem that the human prostate contains very little endogenous EGF (less than 1 ng/g), but the establishment of the EGF receptors in the prostate argues for a role for the ligand in the human BPH tissue. Whether the small quantity of EGF in the prostate is a normal requirement of the prostate, is not known. Furthermore, whether the prostate depends on EGF from another source or not is also not known, but recently Gregory et al (1986) measured large quantities of urogastrone EGF in the human prostatic fluid. Levels found in BPH were half those found in age matched normal controls. The lower level found in BPH is interesting. It is a clear-cut biochemical difference between the two groups. The questions that should be addressed are: Why is the level in the prostatic fluid of BPH lower than in the normal prostatic fluid by as much as 50%? Does EGF in the prostatic fluid diffuse into the prostate tissue to function by interacting with its receptors in the tissue? Whether the high level of EGF seen in the normal prostatic fluid is due to refractoriness of the normal prostate tissue to EGF is not clear. Normal human urothelium has been shown to exhibit refractoriness to EGF (Messing+Reznikoff, 1987). Furthermore, loss of refractoriness of tissue to EGF has been suggested as an early event in the development of dysplasia and neoplasia (Messing+Reznikoff, 1987). It is speculated that in BPH there may be loss of refractoriness

which may now allow the ligand to diffuse into the tissue to interact with its receptor, hence the lower levels of EGF remaining in the BPH prostatic fluid. There is yet another possibility. In a preliminary investigation, it was revealed that the normal prostate expressed no, or very little, EGF receptors. (The source of normal prostate was from whole body donors, aged less than 30 years, but this investigation was curtailed because of the difficulties in obtaining this kind of tissue.) It is possible that the absence of EGF receptors in the normal prostate may be responsible for the lack of effect of EGF in normal tissue. In this regard, any synthesised EGF in the normal prostate may be secreted into the prostatic fluid, but the presence of EGF receptors in BPH may allow some of the synthesised ligand to be retained probably to function there. It is hoped that some of these speculations may help to establish the reason for the difference in the level of EGF-urogastrone between normal and BPH prostatic fluid.

Alternatively, prostatic growth and development may not only depend on EGF but also on other growth factors present in the prostatic tissue which may be functioning in concert with EGF. Recently many workers have described many mitogenic factors isolated from human BPH, rat prostate and adenocarcinoma of the human prostate (Jacob and Lawson, 1980; Story et al, 1983; Jinno et al, 1986; Maehama et al, 1986; Koutsileris et al, 1987). These mitogens were shown to be different from EGF as they all have different physicochemical and biological characteristics from those of EGF and also from one another. They have been implicated in BPH formation. Recently, the impact of multiple growth factors and the multifunctional role of growth factors on tissue growth, development and maintenance has been highlighted (Sporn and Roberts, 1988). It is suggested that the fine tuning

required by adult tissue is provided not by one growth factor but by multiple growth factors and the multifunctional activities of growth factors. The mechanism by which this fine tuning of the tissue is achieved is not clear but may result from interaction between growth factors (Roberts et al, 1985) or growth factor receptor interaction (Bowen-Pope et al, 1983; Zachary+Rozengurt,1985).

It is noteworthy that some of the mitogenic factors described have almost identical characteristics as TGFβ in terms of molecular weight, presence of disulphide bonds and stability in heat and acid (Maehama et al, 1986). The inhibitory effect of TGFβ on fibroblast growth in the presence of EGF is documented (Roberts et al, 1985). Furthermore, the molecular weight of one of the species described as a mitogen in BPH and CaP is 10,000 daltons. This is very close to that of EGF (6,045 daltons) (Koutsilieris et al, 1987). It is possible that on further purification, the 10,000 daltons molecular weight species may turn out to be EGF. These findings give an insight into the nature of mitogenic growth factors that may be functioning in the prostate and therefore may be implicated in BPH and CaP formation. It is likely that these growth factors may include EGF and TGFβ.

Further evidence of the presence of the EGF receptor in the human prostate was achieved by cross-linking and phosphorylation studies. These studies established the molecular weight of the receptor and demonstrated phosphorylation of the EGF receptor in BPH. Affinity labelling, chemical cross-linking with DSS followed by SDS-PAGE analysis of the cross-linked complexes and autoradiography produced a wide band between 150 and 170 KDa molecular weights (Figure 26 Lane A), as determined by molecular weight markers. The specificity of the band was demonstrated by its disappearance in the presence of

excess unlabelled EGF (Figure 26 Lane B). The molecular weight of EGF receptor is known to be 170 KDa (Cohen et al, 1980; 1982), but sometimes a minor protein (150,000 daltons molecular weight) is also observed, which is believed to be a fragment of 170 KDa molecular weight protein produced by proteolysis (Lisley and Fox, 1980; Cohen et al, 1982; Cassel, 1982). EGF receptor expression in the human BPH compared to other tissues (human placenta, rat uterus and human breast tumour etc) is relatively low. Usually only a small proportion of the receptors are crosslinked to $^{125}$I-EGF. This presents a problem when the receptors in the tissue under investigation are low. It was therefore with considerable difficulty that a positive cross-linked EGF receptor complex was demonstrated in this investigation. It is recommended that further work should be done in this area, using BPH tissue which contains much higher EGF receptor levels to improve upon the quality of bands obtained in this report.

However, results are consistent with the findings of earlier workers (Mukku and Stancel, 1985; Ramani et al, 1986; Traish and Wotiz, 1987). Furthermore, the earliest reported response of EGF binding to cell surface receptors is the phosphorylation of its own receptor. Evidence in other systems suggest that EGF binding, the kinase activity and phosphorylation site for the kinase all reside in the same molecule (Cohen et al, 1980; 1982). These observations are consistent with the findings in this report. The addition of labelled ATP to BPH particulate fraction in the presence of EGF, followed by SDS-PAGE and autoradiography revealed that there was phosphorylation of a major protein (molecular weight 170,000 daltons) (Figure 27 Lane A). The presence of the minor protein (molecular weight 150,000 daltons) was not

demonstrated. The molecular weight of the phosphorylation spot was ascertained by the use of molecular weight standards. The phosphorylation experiment was repeated but in the absence of unlabelled EGF. It is noteworthy that the spot of interest appeared only in the presence of (Figure 27 Lane A) but not in the absence of (Figure 27 Lane B) EGF. The reason is not clear, but it seems that in vitro phosphorylation of EGF receptor in BPH only occurred in the presence of exogenous EGF, lending support to the EGF stimulated phosphorylation of its receptor. This investigation also presented identical problems to the cross-linking experiments. Further work is again suggested in this area to improve upon the phosphorylation results.

The presence of EGF receptors in BPH tissue was further confirmed by using monoclonal antibody specific for the EGF receptor site (Waterfield et al, 1982) employing two immunocytochemical techniques. Positive staining of BPH section was indicated by brown colour (Plate 2) or red colour (Plate 4). Positivity which showed the presence of the EGF receptor was confined to the basal layers of the epithelial cells, whilst the stromal areas remained clear (Plates 2, 4). When the use of the monoclonal antibody was omitted no positive staining was observed (Plates 1, 3) indicating that the presence of the monoclonal antibody was a prerequisite to the development of the positive staining reaction.

The localisation of the EGF receptor to the epithelial cells of BPH was in agreement with other reports. McKeehan et al (1984) observed that EGF was implicated in the proliferation of rat prostate epithelial cell populations. This by implication suggested the presence of receptors in the epithelial cells. Furthermore, most recently, Shuurmans et al (1988) measured EGF receptors in the human prostate tumour cell line (LN CaP), an epithelial line. This also

lent support to the fact that EGF receptors were confined to the epithelial cells, but the localisation of EGF to a cell type has not been reported, although EGF has been measured in the prostate (Hirata and Orth, 1979; Traish+Wotiz, 1987). This uncertainty of localisation of EGF means that the mode of EGF action in the prostate still remains undefined. However, the findings of Shikata et al (1984) who localised EGF to the epithelial cells of the guinea pig prostate would seem to suggest an autocrine mode of action for EGF in the prostate. Further work is required to identify the cell type which contains the ligand in the human prostate so as to establish the mode of action. Cunha et al (1986) and Tenniswood (1986) are of the opinion that growth factors are produced in the stromal cells of the prostate but have their proliferative effect on the epithelial cells, suggesting a paracrine mode of action for growth factors in the prostate.

When EGF receptor levels in BPH, determined biochemically, were compared with the corresponding staining intensities, it was realised that there was a good correlation between the biochemical method and the immunocytochemical techniques. Staining scores were in agreement with biochemical results expressed in fmol/mg protein for the corresponding levels of receptors in the human prostate. These findings indicated that the EGF receptor levels could also be assessed by degree of staining intensity.

Using biochemical and immunocytochemical techniques, the presence of EGF receptors was also established in CaP. Scatchard plot analysis revealed only one class of binding sites with only high affinity dissociation constant (Kd) (mean = 1.6 $\pm$ SD 0.4) receptors (Figure 29). The lower affinity receptors seen in BPH (Figure 18) were not observed. It is not clear whether the disappearance of the

low affinity receptors plays any role in the transformation of BPH to well differentiated CaP. Boonstra et al (1985) showed that the presence of low affinity EGF receptors was associated with inhibition of differentiation.

The presence of the EGF receptors was also confirmed by immunocytochemical methods which showed that the receptors were confined to the epithelial cells, whilst the stromal elements remained unstained (Plates 5, 6).

## 4.3 COMPARISON OF EGF RECEPTOR LEVELS BETWEEN BPH AND CaP

The use of biochemical and immunocytochemical techniques was also employed to establish the difference in the receptor expression between BPH and CaP. Clearly BPH expresses more copies of EGF receptors than CaP ($p<0.01$). It is noteworthy that the bulk of the receptors in the CaP is confined to the well differentiated CaP. In this regard, there is no statistical difference between receptor levels in BPH and well differentiated CaP. However, there is a gradual disappearance of the receptor as the tumour becomes less differentiated. This indicates that the receptors are expressed according to the histologic grades of the CaP (Figure 30B). The loss of receptors in the moderately and poorly differentiated CaP is the main reason for the low mean levels observed in CaP compared to BPH (Figure 30A).

These biochemical findings were confirmed using immunocytochemical method which depends on staining intensity as a measure of receptor level. In this regard BPH and well differentiated CaP exhibited the same order of high intensity staining consistent with high EGF receptor levels produced by the biochemical technique (Plates 7, 8, 10, 11), but the intensity started to decrease as the tumour became

less differentiated. Total or near total disappearance of staining was observed when the tumour became poorly differentiated (Plates 9, 12). This intensity varied according to the histologic grade of the tumour. The use of immunocytochemistry had the advantage of demonstrating the neoplastic status of the CaP. The cellular localisation of the receptor and the degree of cellular differentiation were also evident. In well differentiated CaP glandular status is still maintained and comparison with BPH could be easily made. CaP has no basal layers but the staining is still confined to the epithelial cells. In CaP there are more acinar glands and less stromal spaces whereas BPH acinar glands are not as many but are large and there are more stromal spaces. Furthermore, in the moderately and poorly differentiated CaP, the histological derangement is more accentuated. In the poorly differentiated CaP particularly there is a complete loss of glandular formation and no discernible acinar arrangement can be seen.

The intensity of staining may therefore reflect an alteration in the expression of the EGF receptors by the different cellular differentiation stages. Loss of EGF receptors may therefore be associated with loss of glandular formation. Loss of EGF binding could also be explained in terms of presence of v-erb B oncogene product. Comparison of the EGF receptor with the v-erb B oncogene product by molecule cloning (Ullrich et al, 1984) revealed that the v-erb B protein was homologous only with the transmembrane and cytoplasmic domains of the EGF receptor but lacked the majority of the extracellular binding domain. It has been hypothesised that v-erb B protein which cannot bind EGF in a constitutively active state contributes to uncontrolled growth and division of infected cells (Downward et al, 1984).

To test this hypothesis, the human prostate was investigated for presence of v-erb B product or truncated EGF receptor by immunocytochemistry using monoclonal antibody which recognises only the internal domain of the EGF receptor (MAB F4).

Comparison of the staining results on BPH sections (Plates 14, 18) and well-differentiated CaP sections (Plates 15, 19) clearly demonstrate positivity of staining indicating that the monoclonal antibody recognised the internal domain of the receptor, in that staining was mainly cytoplasmic. The poorly-differentiated CaP on the other hand showed a negative staining except for a few isolated areas which showed faint positivity (Plates 16, 20) indicating that the internal domain of the receptor disappeared in the poorly differentiated CaP. The results obtained using both the monoclonal antibodies to external and internal domains of the receptor show that there is total disappearance of the receptor from the poorly-differentiated CaP, the external disappearing faster than the internal domain.

It is noteworthy that the staining picture produced after using the technique of immunoperoxidase involving the use of monoclonal antibody F4 was not in any way clear at all (Plates 13, 14, 15, 16) compared to labelled avidin biotin technique (Plates 17, 18, 19, 20). The immunoperoxidase technique produced an intense non-specific stromal staining which completely overshadowed the epithelial staining, making interpretation difficult. This problem has also been mentioned by Berger et al (1987) after staining lung tumours. Since these authors also used the immunoperoxidase technique, it would appear that the problem is associated with that technique because it is not seen in the labelled avidin biotin method. It will be interesting to investigate this phenomenon.

However, the presence of EGF receptors in BPH and CaP has been recently confirmed (Eaton et al, 1988) but these authors did not observe any correlation between the EGF receptor expression and the histologic grades of the cancer. Furthermore, they observed that CaP tissues expressed more EGF receptors than BPH. The fact that they did not observe any correlation of the receptor level with the histologic grades of the CaP provided an explanation for the higher receptor levels in CaP than BPH which they observed. If low, or no, EGF receptors were observed in moderately and poorly-differentiated CaP respectively, then it should be expected that the mean level of EGF receptors in CaP would be lower than that of BPH. On the other hand, if the moderately and poorly-differentiated CaP expressed equal receptor levels as in well-differentiated CaP, it is unlikely that CaP tissues would have more receptors than BPH. No statistical difference between BPH and well-differentiated CaP was observed in the report of this thesis, although the receptor level is lower in CaP than BPH. Furthermore, another important consideration lies in the selection of pure CaP tissues, for the assay. CaP chips of one particular histologic grade are usually mixed with BPH chips and this may reduce the proportion of cancerous chips in the mixture and decrease one's chances of selecting the correct cancerous chip. The consequence of this is the use of wrong type of tissue. In this regard, it is important to use also immunocytochemical approach to confirm the histologic grade of the CaP. However, the correlation between EGF receptor level and the state of neoplastic progression observed in this report, has also been observed by many workers using a whole range of other tissues or cells. For example, increased expression of receptors was observed in well-differentiated human

squamous cell lung cancers but less in poorly-differentiated counterparts (Hendler+Ozanne, 1984). Comparison of EGF receptor expression between normal and malignant tissues revealed that the bulk of the receptors were associated with the well-differentiated malignant tissues, whilst the normal and poorly-differentiated tumours had only small amounts of the receptors (Gusterson et al, 1984). Furthermore, Libermann et al (1985) also reported increased expression of EGF receptors in well-differentiated primary human glioblastoma whilst the poorly-differentiated tumours had much reduced levels. Associatiion between low receptor level and poor differentiation has also been reported in Chinese hamster embryo fibroblasts (Wakshull et al, 1985). Recently well-differentiated squamous carcinoma cell line was observed to be associated with high levels of EGF receptor (Cowley et al, 1986). These authors observed that the well-differentiated cells possessed ten times more receptor than the poorly-differentiated cells. These findings are in accord with those presented in this report, in that increased EGF receptors were seen to be associated with well-differentiated CaP whilst the poorly-differentiated CaP expressed little or no receptors. On the contrary, however, other workers also observed that high EGF receptor expression was associated with poor differentiation and lymph node metastasis of breast cancer (Sainsbury et al, 1985) and invasiveness of bladder cancer (Neal et al, 1985). These examples indicate that the receptor expression could be used as either a tumour marker or an index of metastatic potential of the tumour, although this may not be the general rule. Hwang et al (1986) did not observe any correlation between EGF receptor expression and the histologic grades of lung cancer, but with regard to gastric carcinoma the picture is unclear. Whilst Sakai et al (1986) did not observe any correlation between EGF receptor levels and the histologic grades of gastric cancer, Yasui et al (1988) were able to correlate EGF and its receptor levels to the

histologic grades of gastric carcinoma. The reason for the difference in the results is not understood. In a recent study, Sainsbury et al (1987) used the EGF receptor status as predictor of early recurrence of, and death from, breast cancer.

The reasons for the high or low EGF receptor expression in the poorly differentiated and metastatic cancers are not clear. However, some possibilities have been put forward to explain. The following are some of the reasons for absence of EGF binding in tissues and cells. It has been shown that binding of EGF to its receptor leads to a decrease in the receptor density on the cell surface (down regulation) as the growth factor receptor complex is internalised (Pastan + Willingham, 1981; Cohen, 1987). Most transformed cells produce transforming growth factor $\alpha$ (TGF$\alpha$) (Salomon et al, 1987) which interacts with the EGF receptor and mimics most of the actions of EGF, i.e. they bind with high affinity to the receptor, stimulate EGF receptor autophosphorylation and also cause down regulation during the process of internalisation (Todaro et al, 1980). This autocrine stimulation renders the transformed cell independent of exogenous EGF requirement. This has been proposed as a model for carcinogenesis.

The implication of the v-erb B oncogene product as another instance of autocrine stimulation of the cytoplasmic EGF receptor has been fully discussed under section 1.4(c).

Androgens have been shown to modulate EGF receptor levels (Traish + Wotiz, 1987). EGF receptor levels in the rat prostate were increased after castration, but levels were reduced when the castrated rats were treated with 5$\alpha$ dihydrotestosterone. The mechanism by which this modulation is achieved is not well understood. Androgens have been shown to down regulate and oestrogens to up regulate LH receptors

in granulosa cells in culture (Jia et al, 1985). These observations suggest that androgens may modulate the response of prostatic cells through regulation of other hormone or growth factor receptors. Androgens are known to elicit the synthesis of growth promoting substances in the stroma which may have their proliferative influences on the adjacent epithelial cells (Cunha et al, 1986). It is tempting to speculate that the presence of androgens may cause the synthesis of large amounts of EGF in the prostate which may down regulate the EGF receptors on the membrane surface, thereby causing their disappearance from the cell surface, but the most recent findings of Shuurmans et al (1988) are at variance with those of Traish et al (1987). Shuurmans et al (1988) observed that androgens increased EGF receptor levels in human prostate tumour cell line (LN CaP). Whether these differences between the two results were due to the type of human cell line used by Shuurmans and associates or to species differences (human and rat), is not clear. This makes the direction of androgen modulation of EGF receptor levels in the prostate unclear.

EGF receptor loss can also occur through other physical factors. The human prostate is known to contain a large amount of proteases (Gotterer et al, 1956; Mann + Mann, 1981; Isaacs and Coffey, 1984). Storage of the prostate tissue is known to aggravate the physical and proteolytic degradation of receptors in the prostate, but storage of BPH tissues and particulate fraction at -70°C for 16 weeks did not alter the EGF receptor levels (Figure 25). This finding is consistent with those of Trachtenberg et al (1981) who did not observe any change in androgen receptor level after 90 days storage of human prostate at -70°C. However, storage of prostate tissue at -20°C resulted in a 50% fall of androgen receptor level after three months (Smith et al,

1983). Since all tissues were stored at -70°C, the loss of EGF receptors in the poorly differentiated CaP could not have been a consequence of storage.

Furthermore, the proteolytic activities of the enzymes present in the prostate may also result in inactivation of the EGF receptor during homogenisation of the tissue and during the incubation period in the course of EGF receptor assay. This may result in loss of EGF receptor, but this possibility was ruled out after establishing the effect of BPH interactions with various enzymes and enzyme inhibitors. $^{125}$I-EGF binding with BPH exhibited different binding characteristics in the presence of various enzymes and enzyme inhibitors. PMSF and leupeptin improved the EGF binding by up to 15% of control. Aprotinin and DNASE however maintained the level of receptors without showing any inhibitory or stimulatory effect. Although trypsin and $\alpha$ chymotrypsin completely inactivated the EGF receptor, a mixture of trypsin and antitrypsin (soybean) improved the binding to the same extent as leupeptin and PMSF (Figure 23). These findings indicate that PMSF, leupeptin and soybean trypsin inhibitor are effective in removing inhibitory substances that may be associated with EGF binding with BPH.

Inclusion of PMSF in the buffer ensures stability of the receptors during tissue processing.

Furthermore, studies concerning influence of pH on EGF binding with BPH showed that pH 7.4 was optimal. At pH 8 and above binding fell sharply to basal levels (Figure 20), whilst low levels were also observed at pH below 7.

These precautionary measures show that loss of receptors seen in the poorly differentiated CaP was not due to any physical factors but is associated with the grade of histologic differentiation of the cancer.

It is also believed that tumour promoters cause inhibition of EGF binding to the target tissues. The mechanism by which this is achieved is not clear, but tetradecanoylphorbol-13-acetate (TPA) for example is thought to activate protein kinase C. By this action the need for $Ca^{++}$ and diacylglycerol for the activation of protein kinase C decreases (Nishizuka, 1984; Cochet et al, 1984; Friedman et al, 1984). Therefore the consequence of either TPA or protein kinase C causes phosphorylation of the EGF receptor. It has been shown that the action of TPA is typical of other tumour promoters (Nishizuka, 1984; Cochet et al, 1984).

Receptor loss has also been associated with lack of gene expression. Molecular evidence has been produced to demonstrate that the lack of EGF receptor in small cell lung carcinoma cells is due to lack of the EGF receptor gene expression in the cells (Gamou et al, 1987). This observation may tempt one to speculate a simular occurrence in the poorly-differentiated CaP where the receptor loss may be a consequence of lack of receptor gene expression.

Further evidence of the link between receptor number and biologic behaviour of the cell was given by a demonstration of the fact that human oesophageal carcinoma cells have fewer but higher affinity EGF receptors (Banks-Schlegel and Quintero, 1986). The mechanisms by which the fewer receptors are expressed is not clear, but may probably be a consequence of receptor gene reduction or down regulation of most of the receptors.

Several lines of evidence have been produced in an attempt to

explain the reason for the increased EGF receptor expression in some cancerous cells and tissues, although the mechanism is still poorly understood. Such increases are usually thought to be due to receptor gene amplification and over expression or gene translocation, amplification and rearrangement. The high receptor levels observed in primary human glioblastoma have been found to result from amplification and over expression of the EGF receptor gene (Libermann et al, 1985). On the other hand, an increase of EGF receptors in epidermoid malignancies has been attributed to receptor gene translocation, amplification and rearrangement (Ozanne et al, 1985). EGF receptors have been shown to increase and cells differentiate in the presence of retinoic acid (Adamson and Rees, 1981). Retinoic acid binding proteins have recently been localised in the human prostate (Boyd, 1985). It may, therefore, play a role in the modulation of the EGF receptors in the BPH and differentiation of the prostatic cells. It is noteworthy that Boyd (1985) observed lower level of retinoic acid in poorly differentiated CaP than in well differentiated counterparts. Glucocorticoids have also been implicated in increase EGF binding. Binding studies indicated that dexamethazone treated cells exhibited a 50 - 100% increase in the ability to bind trace concentrations of $^{125}$I-EGF and this was dependent on the concentration of dexamethazone used (Baker et al, 1978). The mechanism, as explained by Baker et al, (1978), involved conversion of receptors to a form having four-fold increased affinity.

More recently, Shuurmans et al (1988) showed that androgens paradoxically increased EGF receptors in the human prostate tumour cell lines (LN CaP). Contrast this report with that of Traish + Wotiz (1987), who observed that androgens decreased EGF receptor levels,

but such results should be interpreted cautiously. The LN CaP cell lines have been passaged over many years and therefore it is possible that they might have undergone phenotypic changes which may have important implications on the androgen EGF receptor interaction.

The link between high EGF receptor expression and poor differentiation and metastatic potential as observed in the bladder (Neal et al, 1985) and breast (Sainsbury et al, 1985) cancers is not understood but in this case, the phase of the cell cycle has been implicated. The presence of EGF receptors on a cell may be partly a reflection of the means by which it undergoes rapid division. This was demonstrated by the fact that three chemically transformed cell lines showed greater binding of EGF when they were rapidly dividing than when they were arrested in G1 phase by deficiency of nutrients (Robinson et al, 1982), but the three non-transformed parent cell lines showed no increase in EGF binding when they were rapidly dividing (Robinson et al, 1982).

The presence of EGF receptors probably may represent one feature of the genetic alteration that orchestrates the behaviour of the malignant cell. EGF receptor level has been shown to be associated with the capacity of the cell to differentiate. Boonstra et al (1985) observed in normal and transformed keratinocytes that EGF receptor expression was related to the state of differentiation and capacity of cells to differentiate. Furthermore, it was observed that low affinity binding sites increased considerably as cells were less able to differentiate. These observations underline the role of EGF receptors in cancer differentiation.

The reports presented indicate quite clearly that EGF receptor expression may change in any stage of the neoplastic progression. The level expressed may depend upon the type of tissue or cell and the histologic grade and differentiation of the tumour.

It seems therefore that the receptor expression could be a useful tumour marker. Furthermore, it is speculated that the presence of high EGF receptors in BPH may play some role in the transformation of the tissue. The basis of this speculation stems from the fact that EGF receptor expression in BPH and that in well differentiated CaP are almost identical. This follows that those tissues which contain high EGF receptor levels are at risk of being transformed.

This idea is not novel. It has been suggested that elevated numbers of EGF receptors may represent an important step in neoplastic progression (Banks-Schlegel+Quintero, 1986). This is again strengthened by the speculation that increased expression of the EGF receptor above a certain critical level may result in transformation (Downward et al, 1984). Consistent with these two assertions is the view that the chronic production of growth factor at a local premalignant site would precede the immortalising step. This implies that hyperplastic lesions caused by excess of growth factor or its receptor could transform to a neoplastic lesion (Burgess, 1986).

It is interesting to note that the relationships between BPH and cancer of the prostate are traditionally controversial. BPH has been proposed as a direct cause of prostatic carcinoma or as an intermediate state between causative factors and carcinoma (Armenian et al, 1974). However, more recent evidence supports the concept of BPH and CaP as unrelated diseases which may have different pathogenesis rather than being part of the same disease spectrum (Hodges and Wan, 1982; Brendler et al, 1985).

In the light of findings in this report, supported by other reports which have been discussed in this presentation, concerning the status of

EGF and its receptor in carcinogenesis, it is tempting to speculate that there is a direct link between BPH and CaP. This is consistent with the old school of thought that BPH was thought to be a direct cause of prostate cancer as stated above (Armenian et al, 1974). It is the opinion of the author that BPH is transformed to well differentiated CaP, probably due to the presence of relatively high levels of EGF receptor in BPH. In the poorly-differentiated tumour, total disappearance of receptors was observed, probably due to lack of EGF receptor gene expression in the poorly differentiated CaP. Although increased expression of the EGF receptor may play a role in the generation of carcinomas, it may also provide a possible site for adjuvant hormonal or immunotherapy which has become an attractive alternative to standard methods of adjuvant therapy (Gilland et al, 1980; Order et al, 1985; Epenestos et al, 1985). In this regard, experiments were designed to block the binding sites of the EGF receptor using monoclonal antibody to the external domain of the EGF receptor, but these experiments failed to achieve any blocking effect (TABLE 7. Plates 21,22). This may stem from the fact that the monoclonal antibody recognised a site distal to the EGF binding site.

However, monoclonal antibodies are now used in antibody guided immunotherapy in which the monoclonal antibodies are labelled with isotopes or toxins. This method is only used in EGF receptor-positive tissues. Furthermore, the development of monoclonal antibody to the internal domain of the EGF receptor (F4) (Berger et al, 1987) may also argue for a role for the use of the monoclonal in antibody-guided immunotherapy, but these are techniques for the future.

In this report, it has been shown that the human BPH tissue is a target for EGF by the demonstration of presence of EGF receptors which

are confined to the epithelium. The receptors have been shown to be present also in CaP tissues, but the receptor levels in CaP vary according to the histologic grades of the cancer; well differentiated expressing more receptors whilst poorly differentiated express little or none. Furthermore, BPH has more EGF receptors than CaP. The internal domain of the receptor has also been demonstrated. Poorly differentiated CaP has no internal domain.

## 4.4 CONCLUSIONS

In conclusion, the presence of EGF receptors in BPH shows that EGF has a role in the development, maintenance and abnormal growth of the human prostate. Furthermore, the presence and distribution of EGF receptor levels according to the histologic grades of CaP shows that EGF has a role in the development of CaP and the differentiation of the tumour. The absence of truncated receptors in the poorly differentiated CaP shows that the EGF receptors disappear completely with loss of differentiation of the tumour, but whether the loss of the receptors is a consequence of the histologic grade of the tumour, or vice versa, is not clear. When other reports on growth factor interaction with the prostate are considered together with the report presented in this investigation, it is clear that BPH and CaP may not be caused by a single growth factor. Instead, multiple growth factors including EGF and related peptides functioning in concert, and also multifunctional activities of growth factors, may be implicated in the pathogenesis of the two conditions. Interaction between growth factors, and also between growth factors and receptors are thought to be necessary to achieve the fine tuning required by adult tissue. Derangement of these activities may therefore produce undesirable consequences. But the production of these mitogenic

growth factors is believed to be under the influence of androgens (Byyny et al, 1974; Cunha et al, 1986; Tenniswood, 1986). This means androgens have a modulatory role on growth factor activities. Recently, this contention was tested (Traish+Wotiz, 1987; Shuurmans et al, 1988) but the conclusions derived from these studies indicate that the relationship between androgens and growth factors is still nebulous.

## 4.5 FUTURE STUDIES

There are a few areas which need to be investigated and these have been summarised under future studies, as follows:-

1) Measurement of EGF in the human prostate should be made with a view to establishing the mode of EGF action in the human prostate.
2) The identity of the two forms of BPH protein seen in the second linearity during protein measurement and during the heat experiments should be established.
3) The link between the EGF receptors in the human BPH and EGF-uro in the human prostatic fluid should be established.
4) The identity of other mitogenic growth factors present in the prostate should be established.
5) The link between retinoic acid and EGF receptor expression should be clarified.
6) The cross-linking and phosphorylation experiments need to be done again using a prostate tissue containing much higher EGF receptor levels.
7) Characterisation of the proteins of the 800g crude heavy pellet and the microsomal, mitochondrial fractions should be carried out.

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# Localization of epidermal growth factor receptors in the human prostate by biochemical and immunocytochemical methods

**S. Q. Maddy, G. D. Chisholm, R. A. Hawkins* and F. K. Habib**

Department of Surgery, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG
*Department of Clinical Surgery, Royal Infirmary of Edinburgh, Lauriston Place, Edinburgh EH3 9HB

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## ABSTRACT

The receptor for epidermal growth factor (EGF) was characterized in the particulate fraction from human benign prostatic hyperplasia (BPH) and was present in 85% of tissues analysed. The uptake of $^{125}$I-labelled EGF by BPH was dependent on both time and temperature, with maximum specific uptake achieved after incubation for 90 min at 37 °C. Binding characteristics revealed two classes of binding sites of higher (mean dissociation constant ($Kd$) ± S.D. = 0·8 ± 0·2 nmol/l) and lower ($Kd$ = 7·6 ± 2·8 nmol/l) affinities. Competition studies demonstrated the specificity of the receptor assay since the binding of labelled EGF was abolished with excess unlabelled EGF but not with excess unlabelled human GH, human insulin, venom nerve growth factor, human FSH, human LH and human prolactin. There was a complex biphasic relationship between specific binding and protein concentration in the range 0·1–8 mg/ml. Subcellular fractionation of BPH homogenates demonstrated that the bulk of the specific binding was confined to the 800 *g* (crude heavy pellet) and 15 000 *g* (mitochondrial pellet) fractions. The 105 000 *g* (microsomal pellet) and the 105 000 *g* (cytosol fraction) exhibited low and variable binding capacities for the growth factor. The presence of EGF receptor was also confirmed by immunocytochemical staining of frozen sections from BPH using monoclonal antibody specific for EGF receptors. A positive correlation between $^{125}$I-labelled EGF binding and the intensity of staining was found. The presence of a specific EGF-binding receptor protein in human BPH tissues suggests that EGF may play a role in the pathogenesis of human BPH.
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## INTRODUCTION

Mouse epidermal growth factor (mEGF), of molecular weight 6045, was first isolated from mouse submaxillary gland (Cohen, 1962). Human EGF (hEGF; urogastrone; molecular weight 5400) was, however, isolated from human urine (Cohen & Carpenter, 1975; Gregory, 1975). Both mEGF and hEGF exhibit the same intrinsic biological activities and share the same cellular receptor sites in human fibroblasts (Hollenberg & Gregory, 1976; Carpenter & Cohen, 1979). Thus the term EGF may be conveniently used to designate the two peptides.

Epidermal growth factor is a powerful mitogen. It exerts pronounced hypertrophic and hyperplastic effects on epidermal tissue *in vivo* (Cohen & Elliot, 1963) and *in vitro* (Cohen, 1965) and it has also been shown to enhance the growth of fibroblasts (Carpenter & Cohen, 1979). Recent studies have also demonstrated that EGF stimulates proliferation of rat prostate epithelial cell populations (McKeehan, Adams & Rosser, 1984), although its role in human benign prostatic hyperplasia (BPH) remains uncertain (Story, Jacobs & Lawson, 1983).

The biological effects of EGF are thought to be mediated by the interaction with specific plasma membrane receptors in target cells and this has been established in a whole range of tissues (O'Keefe, Hollenberg & Cuotrecasas, 1974; Carpenter & Cohen, 1979; Hollenberg, 1979; Taketani & Oka, 1982). Although earlier reports on the human prostate revealed the absence of EGF and EGF receptors when characterized by biochemical and immunocytochemical techniques (Elder, Williams, Lacey & Gregory, 1978; Gregory, Willshire, Kavanagh *et al.* 1986), other workers have suggested that growth factors might be

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implicated in the pathogenesis of human BPH (Jinno, Ueda, Otaguro *et al.* 1986).

In view of the confusion regarding the status of EGF receptors in the human prostate and their role in the pathogenesis of BPH, we undertook to study in detail the nature of the interaction between BPH and growth factors. We now report the outcome of our investigations which demonstrate the presence of EGF receptors in BPH tissues.

## MATERIALS AND METHODS

### Hormones and growth factors

Mouse EGF and venom nerve growth factor were purchased from Sigma, Poole, Dorset, as electrophoretically pure and were used without further processing. Human prolactin, human follicle-stimulating hormone (hFSH), human luteinizing hormone (hLH), human growth hormone (hGH) and human insulin were generously donated by NIADDK, Bethesda, MD, U.S.A.

### Iodination

Iodination of EGF was by the Iodo-gen method, based on the technique of Fraker & Speck (1978). Briefly, films of 1,3,4,6-tetrachloro-3α,6α-diphenylglycouril (Iodo-gen; Sigma) were prepared by dissolution in methylene chloride and plating on plastic reaction vessels made to react rapidly in the solid phase with aqueous mixtures of $Na^{125}I$ (specific activity 350–600 mCi/ml; Amersham International plc, Bucks) and EGF (5–10 μg) to yield the iodinated peptide. The iodinated peptide was purified by column chromatography (1 × 46 cm) using Sephadex G-50. The percentage bound was calculated after precipitation with trichloroacetic acid. The final specific activity varied between 30 and 70 μCi/μg.

### Other chemicals

The anti-human-EGF-receptor monoclonal antibody (EGF-Rl) was a generous gift from Drs M. D. Waterfield and P. Bennett of the Imperial Cancer Research Fund Laboratories, London. Normal rabbit serum was obtained from Scottish Antibody Production Unit, Carluke, Lanarkshire; peroxidase-conjugated rabbit anti-mouse immunoglobulins were from Dako Ltd, Glostrup, Denmark. Diaminobenzedine and Harris haematoxylin stain were obtained from BDH Chemicals Ltd, Poole, Dorset. All other reagents were of analytical grade except for bovine serum albumin (BSA; Fraction V) which was obtained from Sigma. The following buffers were used throughout the investigation: buffer A containing Tris (10 mmol/l), EDTA (1 mmol/l), EGTA (1 mmol/l), sucrose (0·25 mol/l) and phenylmethylsulphonyl fluoride (0·05 mmol/l), pH 7·4; buffer B containing Tris (10 mmol/l), sodium chloride (0·9%, w/v) and BSA (0·1%, w/v), pH 7·4; buffer C containing Tris (100 nmol/l) and $MgCl_2$ (4 mol/l), pH 5·8; buffer D containing Tris (25 mmol/l), $MgCl_2$ (10 mmol/l), charcoal (0·3%, w/v) and dextran (0·03%, w/v), pH 7·5; buffer E containing $Na_2HPO_4.2H_2O$ (0·05 mol/l), $NaH_2PO_4.H_2O$ (0·05 mol/l) and NaCl (0·9%, w/v), pH 7·4; and buffer F containing Tris–HCl (10 mmol/l), pH 7·4.

### Tissue preparation

Prostate tissue obtained by transurethral resection was transported to the laboratory in ice-cold saline. The tissue was either used fresh or snap-frozen in liquid nitrogen and stored at −70 °C until analysis. Fragments of every prostatic tissue included in the study were examined histologically and in each case BPH was confirmed. Unless specified, all subsequent procedures were carried out at 4 °C. About 1–2 g tissue was washed in buffer A, blotted dry and cut into small pieces. Preliminary dispersion of the tissue was carried out on a microdismembrator (B. Braun A.G., Melsungen, F.R.G.) for 20 s in a prechilled Teflon container. The tissue was further homogenized in 3 volumes of buffer A using an Ystral homogenizer (Scottish Scientific Instruments Centre Ltd, Edinburgh), for two periods of 20 s and 15 s at position 6 with 1-min cooling intervals. The homogenate was subsequently filtered through a metal strainer and the filtrate spun for 40 min at 105 000 ***g***. The resultant pellet (total particulate fraction) was resuspended in buffer B and dispersed further in a glass Dounce homogenizer using 50 strokes with the loose fitting pestle followed by 10 strokes with the tight fitting pestle. The protein concentration of the final total particulate fraction was adjusted to 1 mg/ml and this was used for all subsequent studies except where indicated otherwise. For experiments designed to determine the subcellular distribution of the specific EGF binding protein, the subcellular fractions were prepared according to the method of Leake, Chisholm & Habib (1983) to provide a crude heavy pellet (800 ***g***), the mitochondrial pellet (15 000 ***g***), the microsomal pellet (105 000 ***g***) and the 105 000 ***g*** supernatant fraction (cytosol).

Protein concentrations in tissue fractions were measured by the method of Bradford (1976) using BSA as standard.

### Binding studies

Binding of EGF was determined by a modification of the methods of Edery, Pang, Larson *et al.* (1985) and Sainsbury, Sherbert, Farndon & Harris (1985).

Briefly, homogenate samples were incubated with 6·0 nmol/l (200 000 c.p.m.) $^{125}$I-labelled EGF (specific activity 30–70 μCi/μg) in the presence and absence of a 50-fold excess (300 nmol/l) unlabelled EGF. The final volume of the incubation mixture was 400 μl made up of 200 μl buffer B with or without unlabelled EGF, 100 μl $^{125}$I-labelled EGF and 100 μl sample. Incubation took place at 37 °C and the reaction was terminated by the addition of 1 ml cold (4 °C) buffer B. The samples were harvested by filtration under reduced pressure on glass microfibre filters (Whatman GF/A, Whatman Ltd, Maidstone, Kent) and rapidly washed with 2 × 1 ml aliquots of buffer B. Preliminary studies revealed that two washes were optimal and this was therefore adopted throughout our binding studies. The radioactivity retained by the filter was measured on an LKB gamma counter with an efficiency of 52% and the specific binding was calculated by subtraction of non-specific from total binding.

In another experiment, saturation analysis was performed over a range of 0·5–24 nmol $^{125}$I-labelled EGF/l in the presence or absence of a 50-fold excess of unlabelled EGF at each concentration of $^{125}$I-unlabelled EGF. The dissociation constant (*K*d) and binding sites were estimated by the Scatchard (1949) method. The impact of time and temperature on binding was investigated by incubating homogenate samples as described above at 4, 25 and 37 °C for varying times and the specific binding calculated for each set of conditions. Values are always expressed as means ± S.D.

### Competition studies

Specificity of binding was assessed by incubating $^{125}$I-labelled EGF (6 nmol/l; 36 ng/ml) in the absence and presence of the following unlabelled competitors: hGH, hFSH, human prolactin, human insulin, hLH and venom nerve growth factor, all at a concentration of 1000 ng/ml, and specific binding was calculated.

### Effect of heat and trypsinization

Before incubation with $^{125}$I-labelled EGF, the homogenate sample was pretreated with trypsin (0·05%, w/v), bovine pancrease type I (Sigma Chemicals), at 37 °C for 90 min or heated at 45 °C for 10 min. After one wash the binding was subsequently assessed by incubating the suspension with 6·0 nmol $^{125}$I-labelled EGF/l as described above.

### Pretreatments with $MgCl_2$ and dextran-coated charcoal

Membrane suspension was mixed with 2 ml buffer C at room temperature for 10 min as detailed by Leake *et al.* (1983). The membrane suspensions were subsequently centrifuged at the appropriate ***g*** force and the resultant pellets reconstituted in buffer A.

For experiments designed to determine the effects of dextran-coated charcoal on EGF binding, the membrane suspensions were exposed to 0·2 ml buffer D for 10 min at room temperature. The suspensions were subsequently layered on buffer B and centrifuged at 800 ***g*** for 10 min to remove the dextran-coated charcoal. In all these incubations the specific binding was assessed as described above.

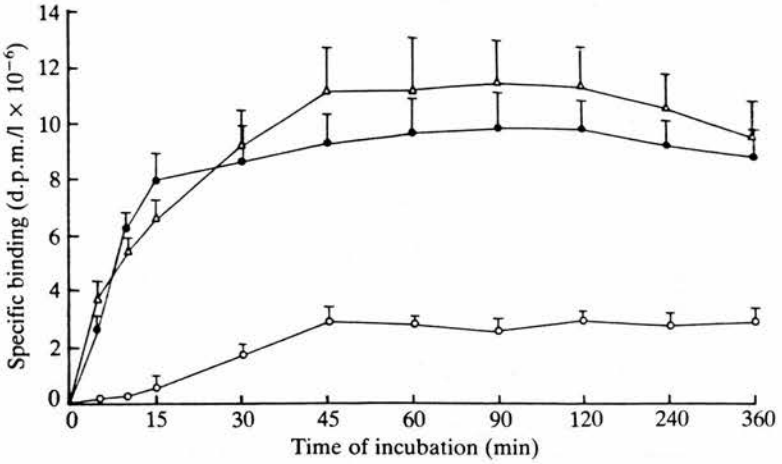
### Immunocytochemical staining with antibodies against EGF receptors

The presence of EGF receptors in BPH was also sought by an indirect immunoperoxidase technique with a murine EGF monoclonal-receptor antibody. Briefly 3–4 μm cryostat sections were cut and collected on lysine-coated slides. After drying with a fan for 30 min the sections were fixed in acetone for 20 min and allowed to dry. Primary antibody (50 μl) at a dilution of 1/30 (v/v) was applied to the sections and these were allowed to incubate for 30 min before being gently washed in Tris–saline for 10 min. A peroxidase conjugate of rabbit anti-mouse immunoglobulin was then applied to the sections for 30 min at room temperature followed by a further 10 min of gentle washing in Tris–saline. The peroxidase enzyme was then visualized by immersion in 0·05% (w/v) diaminobenzedine with hydrogen peroxide for 10 min after which the sections were washed in water. Sections were counterstained with haematoxylin and then dehydrated, cleared and mounted. For each staining experiment a negative control section was included with the primary antibody omitted from the staining procedure.

## RESULTS

### Time and temperature studies

Text-figure 1 illustrates the patterns of binding of EGF to the 105 000 ***g*** membrane pellet at three temperatures. Clearly the binding is time- and temperature-dependent, with maximal binding obtained at 37 °C after 45 min of incubation. The maximal binding was sustained for a further 75 min beyond which a decline was observed, probably due to the instability of the membranes under these conditions. Although the binding at 25 °C was slightly lower than that observed at 37 °C, the binding profiles for both temperatures were similar, whereas at 4 °C receptor–ligand interaction was considerably slower and significantly reduced. It was therefore decided to undertake all subsequent incubations at 37 °C.




TEXT-FIGURE 1. Effect of incubation time and temperature on specific binding of $^{125}$I-labelled epidermal growth factor (EGF) (200 000 c.p.m.; 6 nmol/l) to the total particulate fraction (105 000 *g* pellet) prepared from human hyperplastic prostate. The pellet suspensions were incubated in the presence and absence of unlabelled EGF (50-fold excess) for 90 min at 4 (○), 25 (●) and 37 °C (△). Values are means ± S.D. of six different specimens each analysed in duplicate.

**Effect of protein concentrations on receptor estimations**

In order to determine the limit to which tissue homogenates must be diluted with buffer for accurate receptor estimations, total particulate fraction pellets were resuspended in different volumes of buffer to provide a wide range of protein concentrations. The EGF binding was found to be linear with dilutions corresponding to protein concentrations between 0·1 and 1 mg/ml (Text-fig. 2). As demonstrated below, these protein concentrations provided good quality Scatchard plots, and we therefore restricted all our experiments to this range of concentrations. A second linearity was also observed between 1·5 and 8 mg/ml (data not shown).

**Subcellular distribution**

Subcellular fractions were prepared from specimens obtained from four patients and the specific $^{125}$I-labelled EGF binding was assessed in each component. The data outlined in Table 1 reveal that just over 68% of the specific binding was associated with the 800 *g* (crude heavy pellet) fraction. The remainder of the binding was distributed between the microsomal and the mitochondrial pellets, whereas no specific binding of radiolabelled EGF was detected in the cytosol.

**Affinity and specificity of the binding**

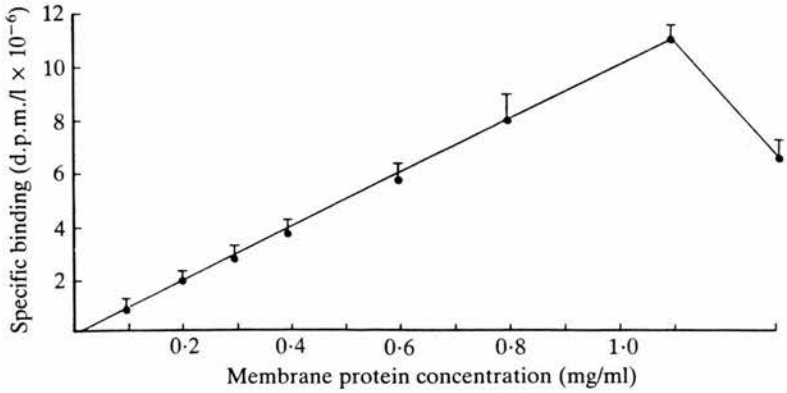
Scatchard analysis of the data from saturation assays carried out on ten separate prostates revealed two binding sites, the first of high affinity ($K$d = 0·8 ± 0·2 nmol/l) and the second of a lower affinity ($K$d = 7·6 ± 2·8 nmol/l). The mean binding capacities for the two components were 14 ± 1·4 fmol/mg protein and 137 ± 23 fmol/mg protein respectively. A typical Scatchard plot for the specific binding to the particulate fraction is shown in Text-fig. 3. The specificity of the binding was also examined. The data outlined in Table 2 demonstrate that human prolactin, human insulin, hFSH, hLH and venom nerve growth factor at concentrations of 1000 ng/ml failed to compete with EGF for the receptor sites whereas hGH (1000 ng/ml) exhibited about 13% competition with the radiolabelled EGF.

**Effect of heat and trypsin**

Pretreatment of the particulate fraction with either protease or heat, before incubation with the radiolabelled ligand, totally inactivated EGF binding (data not shown). The data suggest that $^{125}$I-labelled EGF was bound by a proteinaceous macromolecule associated with the total particulate fractions of the cell.

**Pretreatment with $MgCl_2$ and dextran-coated charcoal**

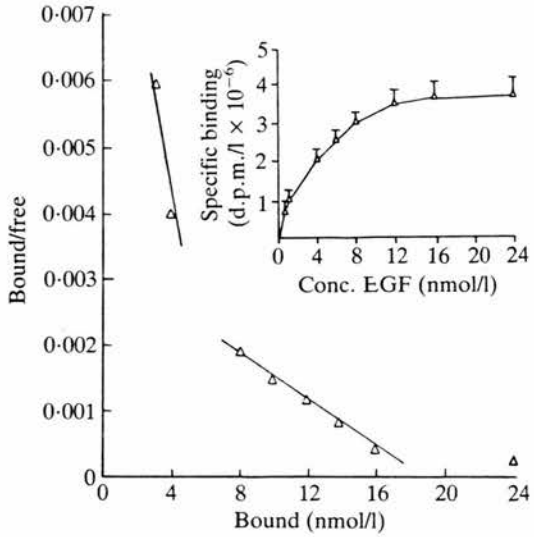
When the particulate fraction was pretreated with either 4 mol $MgCl_2$/l or dextran-coated charcoal, no increase in specific binding was observed. $MgCl_2$ was found to increase both the total and non-specific binding whereas dextran-coated charcoal treatment inhibited these bindings with the overall impact being that of a net loss in specific binding (data not shown).




TEXT-FIGURE 2. Effect of protein concentration of the total particulate fraction (105 000 *g* pellet) on the estimation of epidermal growth factor (EGF)-receptor levels in human prostate. Aliquots (100 µl) of the suspension containing 0·1 to 8 mg protein/ml were incubated with $^{125}$I-labelled EGF (200 000 c.p.m.; 6 nmol/l) in the presence and absence of a 50-fold excess of unlabelled EGF for 90 min at 37 °C. Values are means ± S.D. of four different specimens each analysed in duplicate.

TABLE 1. Subcellular distribution of epidermal growth factor (EGF) receptors in benign hyperplastic prostate. Pellets, resuspended in 100 µl buffer containing Tris (10 mmol/l), sodium chloride (0·9%, w/v) and BSA (0·1%, w/v) (pH 7·4) were incubated with $^{125}$I-labelled EGF (6·0 nmol/l) at 37 °C for 90 min in the presence and absence of unlabelled EGF (50-fold excess). Each value represents the mean ± S.D. of four different specimens, each analysed in duplicate

| | Specific binding (d.p.m./l × $10^{-6}$) | Percentage of total binding |
|---|---|---|
| **Fraction** | | |
| 800 *g* crude pellet | 11·82 ± 1·73 | 68 ± 4 |
| 15 000 *g* pellet (mitochondria) | 3·65 ± 0·53 | 21 ± 5 |
| 105 000 *g* pellet (microsome) | 1·88 ± 0·25 | 10 ± 1 |
| 105 000 *g* supernatant (cytosol) | 0 | 0 |




TEXT-FIGURE 3. Saturation curve and Scatchard plot of specific $^{125}$I-labelled epidermal growth factor (EGF) binding to human prostates. Aliquots (100 µl) of the total particulate fraction (105 000 *g* pellet) were incubated with $^{125}$I-labelled EGF (0·5–24 nmol/l) in the presence and absence of a 50-fold excess of unlabelled EGF. The specific binding data was analysed by the Scatchard (1949) method.

## Immunocytochemical studies

The pattern of immunoperoxidase reactivity observed in BPH is illustrated in the Plate. Staining by the antibody was confined only to the basal layers of the epithelial cells whilst the adjacent stroma remained clear. There was no apparent nuclear staining but the immunoperoxidase fraction was limited to cell membranes. Staining of epithelial cells was predominantly uniform throughout the same specimen but there was considerable variability in the intensity of staining between different tissues. In general, there was very good correlation between the intensity of staining and receptor positivity as measured by ligand exchange assay. Staining of sections was assessed by three independent observers and graded as negative, moderate or intense. Agreement between the two techniques occurred in all ten tissues examined: six specimens demonstrated intense immunocytochemical staining and this corresponded to high specific binding (> 6 d.p.m./l × $10^{-6}$) whereas moderate or negative staining corresponded to binding less than 6 d.p.m./ l × $10^{-6}$.

TABLE 2. Specificity of $^{125}$I-labelled epidermal growth factor (EGF) binding to benign hyperplastic prostate tissue membranes. The particulate fraction was resuspended in buffer B containing Tris (10 mmol/l), sodium chloride (0·9%, w/v) and BSA (0·1%, w/v) (pH 7·4) and 100 µl of the suspension was incubated with 6·0 nmol $^{125}$I-labelled EGF/l (approx. 36 ng/ml) at 37 °C for 90 min in the presence and absence of the competitor (1000 ng/ml). 100% competition was taken as the amount of $^{125}$I-labelled EGF displaced by 1000 ng unlabelled EGF/ml. Each value represents the mean ± S.D. of four different specimens, each analysed in duplicate

| | Specific binding (d.p.m./l × $10^{-6}$) | Competition (%) |
|---|---|---|
| **Unlabelled hormone** | | |
| Venom nerve growth factor | 3·34 ± 0·3 | 4 |
| Human GH | 10·88 ± 1·2 | 13 |
| Human prolactin | 3·38 ± 0·8 | 4 |
| Human FSH | 3·36 ± 0·4 | 4 |
| Human LH | 3·50 ± 0·9 | 4 |
| Human insulin | 3·30 ± 0·6 | 4 |
| Mouse EGF | 83·69 ± 8·2 | 100 |

## DISCUSSION

The results presented in this study suggest for the first time that BPH tissues contain receptor proteins with a high affinity and low capacity for EGF. The characteristics of these receptors were similar to those established for other EGF receptors measured in human placenta (Hock & Hollenberg, 1980), normal mammary cells (Taketani & Oka, 1982), human breast cancer (Sainsbury *et al.* 1985), human bladder cancer (Neal, Bennet, Hall *et al.* 1985), human fibroblasts (Carpenter & Cohen, 1979) and human cervical and ovarian cancer (Gullick, Marsden, Whittle *et al.* 1986).

In common with other reports (O'Keefe *et al.* 1974; Hofmann, Rao, Barrows *et al.* 1984), our binding data for EGF were found to be essentially biphasic since Scatchard plots yielded two classes of binding sites. The presence of multiple binding sites has been attributed primarily to the heterogeneity of the tissue under investigation (Hofmann *et al.* 1984). In a recent study on whole mammary gland membranes, two sets of independent receptor sites were identified (Edery *et al.* 1985) whereas only one class of receptors was demonstrated in isolated epithelial cells obtained from the same gland. Since the prostate also consists of a mixture of glandular and stromal tissues, it is conceivable that the heterogeneity of the specimen analysed could be responsible for the curvilinear Scatchard plots obtained in this study. However our immunocytochemical investigations suggest the absence of EGF receptors in the stroma.

Contrary to earlier findings (Hock & Hollenberg, 1980) pretreatment with $MgCl_2$ and dextran-coated charcoal did not appear to affect the binding of EGF to its receptor. $MgCl_2$ has been shown to dissociate tightly bound endogenous ligands from their receptors whilst dextran-coated charcoal removes possible binding inhibitors from the tissue extracts (Kelly, Leblanc & Djiane, 1979). Our findings may therefore reflect the possibility that BPH contains little endogenous EGF; this is supported by the recent findings of Gregory *et al.* (1986) who failed to show the presence of EGF within the cells of the BPH prostate by immunocytochemical methods. The other possibility is that the binding between the EGF and its receptors in the prostate is not so strong as that observed in other target tissues and this might allow for a complete and effective exchange between endogenous and exogenous ligands; it is notable though that the mean *K*d values measured in the present study were in the region of 0·8 nmol/l and much higher values were found in other cells (0·53–0·007 nmol/l; Boonstra, de Laat & Ponec, 1985).

It is quite evident from our subcellular distribution experiments that the bulk of the EGF binding (68%) was associated with the 800 *g* pellet which incorporates the cell membrane along with the EGF receptors. Further confirmatory support for the presence of these receptors stem from our immunocytochemical staining experiments which revealed their presence in epithelial basal cells. The intensity of the immunocytochemical staining corresponded with the number of binding sites measured by the ligand binding assay. The localization of the receptors on the epithelial cells is in line with recent reports of the ability of EGF to stimulate rat prostate epithelial cells (McKeehan *et al.* 1984). However, our inability to demonstrate any EGF binding along the stromal components of the prostate tissue is at variance with the observations implicating the action of EGF on fibroplastic proliferation in the development of BPH (Jinno *et al.* 1986). Nonetheless, at this stage we cannot discard the possibility that an EGF-like growth factor, synthesized by the stromal component of the prostate, is in some way implicated in the growth and proliferation of the gland. It is noteworthy that in recent studies a number of workers have identified prostatic growth factors in homogenates of human BPH (Story *et al.* 1983; Jinno *et al.* 1986); whether the components are similar to EGF remains to be established.

## ACKNOWLEDGEMENTS

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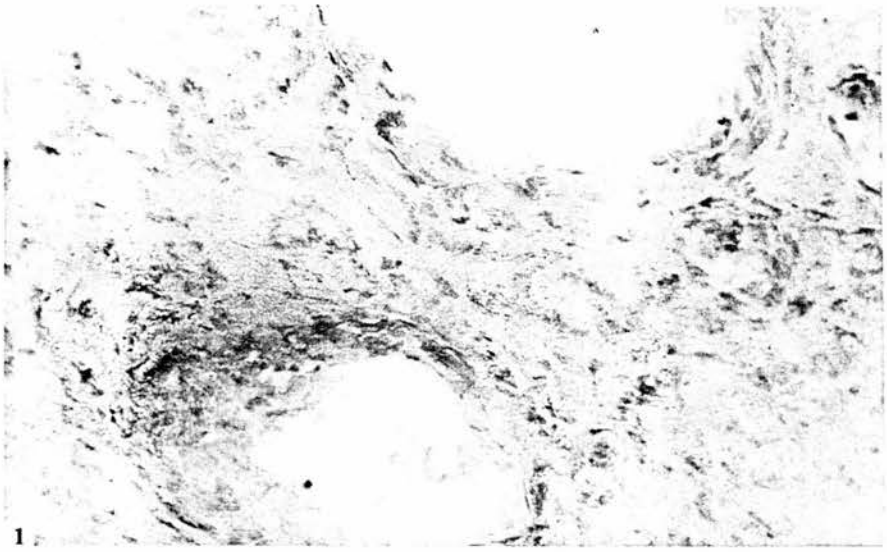

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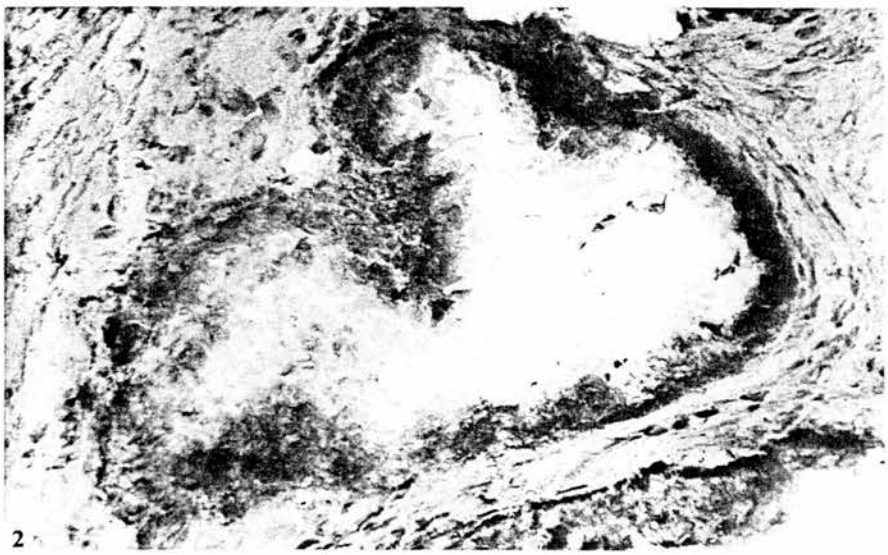

2

## DESCRIPTION OF PLATE

Immunoperoxidase staining of frozen sections of human benign prostatic hyperplasia. ( × 320.) Primary monoclonal epidermal growth factor-receptor (EGF-Rl) antibody was used at a dilution of 1:30. The sections were lightly counterstained with haematoxylin. A positive reaction for EGF-Rl appears as a dark stain along the basal epithelial cells.

FIGURE 1. Section processed in the absence of the primary antibody.

FIGURE 2. Section incubated with EGF-Rl monoclonal antibody.

(Facing p. 154)

# ARE EPIDERMAL GROWTH FACTOR RECEPTORS PRESENT IN THE HUMAN PROSTATE

FOUAD K. HABIB and SAMUEL Q. MADDY

*Department of Surgery, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, Scotland, U.K.*

ABSTRACT

The identification and characterisation of epidermal growth factor (EGF) receptors in hyperplastic and malignant human prostate is described. Two methods were employed: A biochemical technique using a ligand exchange assay and an immunocytochemical approach employing a monoclonal antibody to the EGF receptor.

KEYWORDS

Epidermal growth factor - receptors: immunocytochemical staining: benign prostatic hyperplasia: cancer of the prostate.

## INTRODUCTION

Epidermal growth factor (EGF) is a low molecular weight polypeptide found in high concentrations in human urine and prostatic fluid (Gregory et al, 1986). Its physiological function is not clear, although it has been shown that EGF may induce some biological effects both in vivo and in organ culture by promoting proliferation of the basal cell layers of various epithelia of ectodermal origin (Cohen and Taylor, 1974). It has also been reported that EGF may stimulate the growth and proliferation of rat and human prostate epithelial cell populations (McKeehan et al, 1984; Chaproniere and McKeehan, 1986). These prostatic responses are probably induced following the interaction of the growth factor with specific plasma membrane receptors located on the surface of the human prostate cell (Maddy et al, 1987).

Although no one has so far investigated the relative expression of these receptors in prostate specimens obtained from patients with different pathological conditions, the earlier reports on prostate fluid (Gregory et al, 1986) suggest that EGF secreted by the hyperplastic prostate (BPH) is approximately half that of age-matched controls. It therefore appears that the secretion of EGF by the human prostate is in part dependent on the pathophysiology of the gland but whether this would also result in a parallel change in the concentrations of tissue EGF-receptors remains to be established.

However it is worth noting that in the earlier studies on human lung tumours (Hwang et al, 1986) and human gastric cancer (Sakai et al, 1986) it was shown that the binding activity of EGF to the receptor was either decreased or increased when compared to normal tissues depending on the type of tissue examined.

We now wish to extend some of these studies to the human prostate and compare the levels of EGF receptors in benign and cancerous tissues. Attempts will also be made to assess the impact of histological differentiation in cancer on the expression of these receptors.

## MATERIALS AND METHODS

### Human tissues

Prostate specimens were taken from 18 patients with benign prostatic hyperplasia (BPH) and 19 patients with cancer of the prostate (CaP). None of the patients from whom the tumour samples were obtained received chemotherapy or radiation prior to the removal of their tissue. Histopathologically, the cancerous specimens consisted of 4 well differentiated, 10 moderately differentiated and 5 poorly differentiated tumours. Grading has also been carried out by a single pathologist according to the Gleason system (Gleason and Mellinger, 1974). The specimens were immediately frozen in liquid nitrogen and kept at -70°C pending analysis.

### Measurement of EGF receptors (Ligand exchange assay)

The receptor binding assay was essentially the same as previous described (Maddy et al, 1987) with minor modifications. Following the incubation of the 105000g membrane preparation with the $^{125}$I-labelled derivatives of mouse EGF, the $^{125}$I-EGF-receptor complex was precipitated with equal volume of 20% polyethylene glycol 8000 (PEG) containing 0.1% bovine serum albumin (BSA) at 4°C for 10 min. The mixture was centrifuged for 20 min at 3000 rpm and the resultant pellet was washed once with 10% PEG and 0.05% BSA and centrifuged again as before. The bound radioactivity in the final pellet was measured on a Gamma counter.

### Immunohistochemical studies

3-4 um thick frozen prostate sections were cut on a cryostat. Sections were transferred to microscope slides coated with lysine, fixed in acetone for 20 min and allowed to dry. Sections were subsequently incubated at room temperature with a murine monoclonal antibody (EGFR-1; donated by Dr. M. Waterfield) at a dilution of 1:30 (v/v) for 30 min followed by a peroxidase conjugate of rabbit anti-mouse immunoglobulin and finally the hydrogen peroxide and diaminobenzidine with tris-saline washes between each stage. Rabbit serum obtained prior to the immunisation schedule served as a control and tris-saline as a blank by replacing EGFR-1 in the incubation sequence. The intensity of the staining of each prostate section was assessed by 3 independent investigators using reference slides. The scores for staining ranged from 1+ (weak staining) to 3+ (intense staining); 1- represents a negative reaction. Details of these procedures have been described in our previous paper (Maddy et al, 1987).

## RESULTS

The binding of $^{125}$I-labelled m-EGF to human prostate membrane preparations was found to be saturable and biphasic since Scatchard plots yielded 2 classes of binding sites (data not shown); the first of high affinity $K_d$ = 0.8 + 0.2 nmol/l and the second of a low affinity $K_d$ = 7.6 + 2.8 nmol/l. These binding sites were essentially proteinaceous in composition since pretreatment of the particulate fraction with either trypsin (0.05%; w/v) or heat (45°C; 10 min) prior to the incubation with $^{125}$I-labelled EGF totally abolished the binding. Furthermore our investigations indicated that the interaction between the ligand and the receptor protein was specific to EGF because excess unlabelled competitors (1000 ng/ml) such as nerve growth factor, human growth hormone, human prolactin, human FSH, human LH and human insulin totally failed to inhibit the EGF binding.

Analysis of our receptor measurement data on 18 hyperplastic prostates revealed an average of 125 fmol of $^{125}$I-labelled EGF/mg protein (Table 1) (range: 0-360 fmol/mg protein). In contrast the preliminary data on 19 patients with cancer of the prostate demonstrated a marked reduction in specific EGF binding (mean + SD = 50 + 11 fmol/mg protein; range: 0-120 fmol/mg protein). In spite of an overlap between the 2 groups there was a statistically significant difference between the mean levels of

Table 1. EGF-RECEPTORS: COMPARISON OF BIOCHEMICAL AND IMMUNOCYTOCHEMICAL METHODS IN BPH AND CANCER OF THE PROSTATE.

| Tissue Type | Gleason Score | Mean Specific Binding ± S.D. (fmol/mg protein) | Staining Intensity | Positive Staining |
|---|---|---|---|---|
| BPH | ---- | 125 ± 20 | - / +++ | 17/18 |
| | 2-4 | 84 ± 13 | + / ++ | 4/4 |
| Cap | 5-7 | 52 ± 8 | - / ++ | 5/10 |
| | 8-10 | N.D. | - / - | 0/5 |

EGF-receptors in BPH and cancer of the prostate ($p<0.01$). Although the bulk of the benign prostates assayed for EGF-receptors were found to be positive, this was not the case in malignancy where 10/19 glands were devoid of EGF binding or at least below the detection limits of the assay (12 fmol/mg protein).

In addition to the differences observed between BPH and cancer of the prostate, we were also able to detect a strong relationship between the levels of EGF receptor binding and histological differentiation in cancer (Table 1). In fact, well differentiated tumours (Gleason sum 2-4) maintained significantly larger ($p<0.01$) concentrations of EGF receptors (mean ± SD = 84 ± 13 fmol/mg protein) than in the poorly differentiated tumours (Gleason sum 8-10) which were devoid of EGF binding. The receptor concentrations within the moderately differentiated tumours (Gleason score 5-7) were of an intermediate range (52 ± 8 fmol/mg protein) but nonetheless considerably lower than those measured in the well differentiated tumours.

The presence of EGF receptors in BPH and cancer was also confirmed by immunocytochemical staining. Staining by the antibody was confined only to the basal layers of the epithelial cells whilst the adjacent stroma remained clear. Although the staining of the epithelial cells was predominantly uniform throughout the same specimen, there was considerable variability in the intensity of staining between different tissues and particularly amongst the cancer specimens (Table 1) where well differentiated tumours demonstrated intense immunocytochemical staining (3+) whereas poorly differentiated tumours showed no staining whatsoever (negative). Furthermore, there was a very good correlation between the intensity of staining and receptor positivitity as measured by ligand exchange assay (Table 1). Agreement between the 2 techniques occurred in all 37 specimens examined.

## DISCUSSION

Data presented in this report suggest that the human prostate has an ability to specifically bind EGF. The presence of EGF receptors on the epithelial cells of benign prostatic hyperplasia and cancer of the prostate was initially established by a ligand exchange assay and subsequently confirmed by immunocytochemical staining employing a monoclonal antibody to the EGF-receptor. Furthermore our study reveals that malignant prostates have a decreased ability to bind EGF when compared with the benign hyperplastic gland. Suppressed EGF was also found in a number of other human malignancies including gastric (Sakai et al, 1986) and liver (Costreni and Beck, 1983).

These data were interpreted to mean fewer copies of EGF receptors/cell although no evidence was presented to support such a contention. The possibility exists that decrease binding of radiolabelled ligand may reflect other factors e.g.:(1) Alteration of the EGF receptor molecule with a reduced binding affinity for ligand. (2) The presence of interfering endogenous EGF. (3) Enzymatic inactivation of EGF. (4) The production of a TGF which can down regulate the receptor by the transformed cells. In this study we found no alteration in the dissociation constant ($K_d$) for EGF binding in prostate cancer (data not shown). Thus, it would seem unlikely that endogenous ligand and/or alteration in the EGF receptor molecule can account for our observations of suppressed binding in malignancy. Also since various enzyme inhibitors including anti-trypsin did not augment radioligand binding in the prostate membrane fractions (data not shown), it is unlikely that the difference in EGF receptor values between both sets of tissues is a consequence of enhanced activation of the macromolecule in prostate cancer. For these reasons the possibility that prostate EGF receptors is being expressed in reduced amounts in cancerous tissue must be entertained and as indicated above such a concept is not novel.

In view of the reduced levels of EGF receptors in malignant tissue compared to BPH, it was not surprising to find a correlation between the levels of these binding sites and the histological differentiation of the tumour. To the best of our knowledge this is the first time that such a correlation has been established in cancer specimens and though it is very difficult at this stage to assess the aetiological significance of these findings, the possibility exists that these data might be of prognostic significance. In this regard low EGF binding in patients with BPH could be used to identify those who, in time would progress to a malignancy. Furthermore the use of an EGF receptor cDNA probe could provide further information with respect to the genomic organisation of the EGF receptor gene and the level of the expression of these genes in these tumours as compared to normal and benign prostates.

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